RUSH-PRESBYTERIAN-ST. LUKE'S

MEDICAL BULLETIN

VOL. 15, NO. 1 JANUARY 1976

Rush-Presbyterian-St. Luke's Medical Bulletin
is published quarterly by the staff of
Presbyterian-St. Luke's Hospital, Rush Medical
College, and the Alumni Foundation.

All correspondence relative to the publication of papers should be addressed to the Editor, Rush-Presberterian-St. Luke's Medical Bulletin, Room 242, 1725 West Harrison Street, Chicago, Illinois 60612. All other correspondence should be addressed to Rush-Presbyterian-St. Luke's Medical Bulletin, Room 1007, 1725 West Harrison Street, Chicago, Illinois 60612. Requests for reprints should be sent directly to the authors.

VOL. 15, NO. 1 JANUARY 1976

TABLE OF CONTENTS

A STRESS ANALYSIS OF THE FEMORAL STEM IN TOTAL HIP PROSTHESES

Thomas P. Andriacchi
Jorge O. Galante
Theodore B. Belytschko
Steven Hampton

ABSTRACT. Stresses in the femoral stems of total hip prostheses were studied using an analytical finite element model and in vitro experimental methods. The analytical model was used to study the effects of the following parameters on stresses in the femoral stem: orientation of the stem, angular changes in the hip joint force, integrity of the cement envelope, stem design, and calcar support. The results of two in vitro experiments are in agreement with the predictions of the analytical model.

The stress distribution in the femoral stem is found to result primarily from a bending mode of deformation, with the largest stresses occurring along the middle portion of the stem length.

Cyclic stress reversals in the femoral stem are shown to result from angular changes in the resultant force at the hip joint. Support of the stem at the calcar has a significant effect on the magnitude of stresses in the femoral stem. Stresses in the femoral stem vary inversely with the stiffness of material supporting the stem at the calcar.

INTRODUCTION

Total hip joint replacement is commonplace today in the surgical treatment of hip joint disabilities. Recent reports indicate that mechanical failure of the femoral stem must be included among the possible complications of this procedure. Since fractures of the femoral stem probably result from cyclic stresses beyond the endurance limit of the implant material, an understanding of the interrelated effects of the surgical mechanics of implantation, prosthetic design, and prosthetic materials is crucial to the solution of this problem. It is the purpose of this study to apply analytical and experimental methods to the study of some of the factors affecting stress levels in femoral stems of different designs.

From the Department of Orthopedic Surgery, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois, and the Department of Material Engineering, University of Illinois, Chicago Circle, Chicago, Illinois

Thomas P. Andriacchi, Ph.D., Assistant Professor, Department of Orthopedic Surgery and Biomedical Engineering, Rush Medical College

Jorge O. Galante, M.D., Senior Attending and Chairman, Department of Orthopedic Surgery, Presbyterian-St. Luke's Hospital; Professor of Orthopedic Surgery, Rush Medical College, Chicago, Illinois

Theodore B. Belytschko, Ph.D., Associate Professor of Material Engineering, University of Illinois Chicago Circle, Chicago, Illinois

Steven Hampton, M.S., Research Assistant, Department of Material Engineering, University of Illinois, Chicago Circle

REVIEW OF FEMORAL STEM FAILURES

A summary of data for eleven failed femoral stems is given in Table I. Six of these failures were reported by Galante, et al.[1] An additional five were observed subsequently. Fatigue failure, occurring an average of 35 months after implantation, was the cause of stem fracture in all eleven cases. The average failure location site was 68.4 mm from the proximal lateral shoulder, measured along the lateral surface. The range was from 56 to 80 mm.

TABLE I
SUMMARY OF ELEVEN FEMORAL STEM FAILURES

Prosthesis Stem Design	Fracture Location*	Time to Failure Postoperatively (Mo.)	Patient Age (Years)
Charnley, Standard	66	19	58
Charnley, Standard	65	40	70
Charnley, Standard	73	29	63
Charnley, Straight Stem	56	61	67
Mueller, Short Neck	56	10	80
Mueller, Short Neck	68	24	61
Mueller, Standard Neck	80	27	61
Mueller, Standard Neck	56	48	56
Mueller, Long Neck	69	4	72
Mueller, Long Neck	86	50	71
Mueller, Long Neck	77	36	67

*Distance measured from shoulder along the lateral stem curvature.

Thus, all failures occurred in the middle third of the stem. The mean failure location with a Charnley stem was 65 mm, and for the Mueller stems was 70.3 mm. Although these data are not yet statistically significant, they appear to indicate that the mean failure location for Charnley stem design was more proximal (65 mm) than the Mueller stem (70.3 mm).

Martens, et al.[2] reported a study of six fatigue failures in stems of Charnley-Mueller type. The clinical findings are similar to those reported in Table I. Although the average failure time of the stem was somewhat shorter (between 18 and 27 months), the fractures were all located in approximately the middle third of the stem, between 36 percent and 71 percent of the total stem length. Charnley,[3] in a clinical study, reported the common features of 13 typical failed femoral stems. He cited failures from 16 to 60 months after implantation, with a median time of 40 to 48 months. Typical fracture locations were again reported to be in the middle third of the stem.

There are two possible causes for the early fatigue failures described above; (1) stresses which exceed the endurance limit of the implant material, and (2) metallic defects. In the studies cited, fractures consistently occurred in the middle third of the stem, so it is reasonable to conclude that the stresses here are high. It is unlikely that metallic flaws would be present only in this region.

Therefore, an understanding of the factors affecting stress levels in the femoral stem appears to be a necessary first step towards eliminating the possibility of stem fractures. Once the level of stresses sustained by the femoral stem is understood, quality standards for implant materials can be established.

A femoral stem implanted in the femur is a three-dimensional solid composed of cortical bone, cement, metal, and trabecular bone. The complexity of the problem precludes rigorous analysis by classical methods. The finite element method is well suited to biomechanical studies of this type, and has been successfully used by a number of investigators for biomechanical studies. For example, Rybicki, et al.[4] used a two-dimensional finite element model for studies of stresses in the human femur. Belytschko, et al.[5] reported an axisymmetric finite element stress analysis of the intervertebral disc. However, even with powerful methods such as finite element techniques, the stress analysis of an implanted prosthesis still requires many substantial assumptions and simplifications. Thus, while three-dimensional finite element methods are available, they are expensive, require large computers, and in many cases, may not provide improved solutions. Therefore, a two-dimensional model was used for this study.

The analytical model was used to study, quantitatively, the effects of the following parameters on stresses in the femoral stem: angular changes in the hip joint force, integrity of cement envelope, stem design, and calcar support. To examine the suitability of this model, *in vitro* experimental results were obtained and compared with results predicted analytically.

MATERIALS AND METHODS

Analytical Model

The two-dimensional finite element model used for the stress analysis of the femoral stem includes a representation of the stem, surrounding cement and proximal half of the femur. Since a two-dimensional model was used in this study, only the cement and bone lying in the plane of the stem were included. The validity and limitations of this approximation were examined as part of this study. Details of the finite element methods can be found in standard textbooks such as Meek[6] or Zienkiewicz.[7] To model an object for stress analysis by finite elements, it is divided into a number of discrete regions. The governing equations for each discrete region are assembled into a system of simultaneous linear equations. The equations are solved on a digital computer and the resultant stresses throughout the solid can be determined. The method is very useful in stress analyses of the type reported here, since it is capable of dealing with complex geometries as well as composite materials. The elements chosen for this study were constant strain quadrilaterals and constant strain triangles. These elements make it possible to generate an accurate representation of an implanted stem and still maintain an element aspect ratio necessary for accurate calculations. A complete description of the limitations and implications of this model for the study of implanted prostheses is beyond the scope of the report. A detailed discussion of the technical aspects of implant stress analysis, including both two-dimensional and three-dimensional finite element analysis, as well as the development of simpler beam models, is reported in Andriacchi, et al.[8]

Physical Property Data

The finite element model requires three types of data as input: material properties, geometrical configuration, and external force. Representative material properties were assigned to the cortical bone, trabecular bone, cement and metallic portions of the model. The elastic property data for bone was derived from recent reports by Reiley,[9] Bonfield,[10] and Pope;[11] the properties for bone cement were obtained from Lautenschlager.[12] Material properties are given in Table II. All materials in the model were approximated as isotropic, elastic, and homogeneous materials. This is, of course, an approximation for bone and cement. However, since the primary purpose of the model was to study stresses in the stem, this assumption did not seem unreasonable.

TABLE II
MATERIAL PROPERTY DATA

Material Type	Young's Modulus (E) (Nt/cm^2)	Poissons Ratio (v)
Stainless 316	1.96×10^7	.29
Cortical Bone	1.76×10^6	.30
Trabecular	2.8×10^4	.30
PMM	2.3×10^5	.23

The geometrical data consisted of a set of coordinates describing the relative shape of the stem, surrounding cement and femur. The coordinate data were taken from radiographs of implanted femoral stems, so that representative geometries of the cement and bone could be examined. Radiographs of four implanted femoral stems were digitized; three Charnley stems in a varus, valgus and neutral position, and one Mueller stem in a neutral position. Data taken from the radiographs were scaled on a basis of the stem length and height. Between four and six hundred coordinates were taken from a single radiograph. Computer-generated plots of the digitized radiograph are shown in Figs. 1 and 2.

A unit load was applied to the femoral head in three load directions; 0°, 20° and 45° with respect to the longitudinal axis of the femur as illustrated in Fig. 3. The actual loads transmitted at the hip joint have been reported by a number of investigators including Rydell,[13] Paul,[14] and Williams.[15] Their studies indicate that the magnitude of the typical load at the hip joint can attain four to six times body weight. In addition, the direction of the resultant force is constantly changing during various activities. Since linear elastic behavior is assumed in this study, and be-

cause the stress distributions are reported in terms of stress per unit load, stresses for any force between 0° and 45° applied to the femoral head can be obtained by a superposition of the reported stresses.

In-Vitro Validation Studies

Since the analytical model involved a number of untested assumptions, an experimental study was also undertaken. Two Charnley femoral stems were instrumented with foil micromeasurement strain gauges, as follows. The first stem had two gauges on the lateral surface and two on the medial surface, as shown in Fig. 4. The distal gauge on the medial surface was damaged during implantation. The second stem had four gauges along the lateral surface. Gauge locations are shown in Fig. 3. All gauges were aligned to measure longitudinal stresses along the lateral and medial surfaces of the femoral stem. Gauges were cemented in place with Micromeasurement M-Bond 610 Epoxy cement. The instrumented prosthesis was placed in an oven for one hour at 230°C. to allow the cement to cure; this procedure was needed to insure the integrity of the gauge against heat generated by the bone cement at the time of implantation.

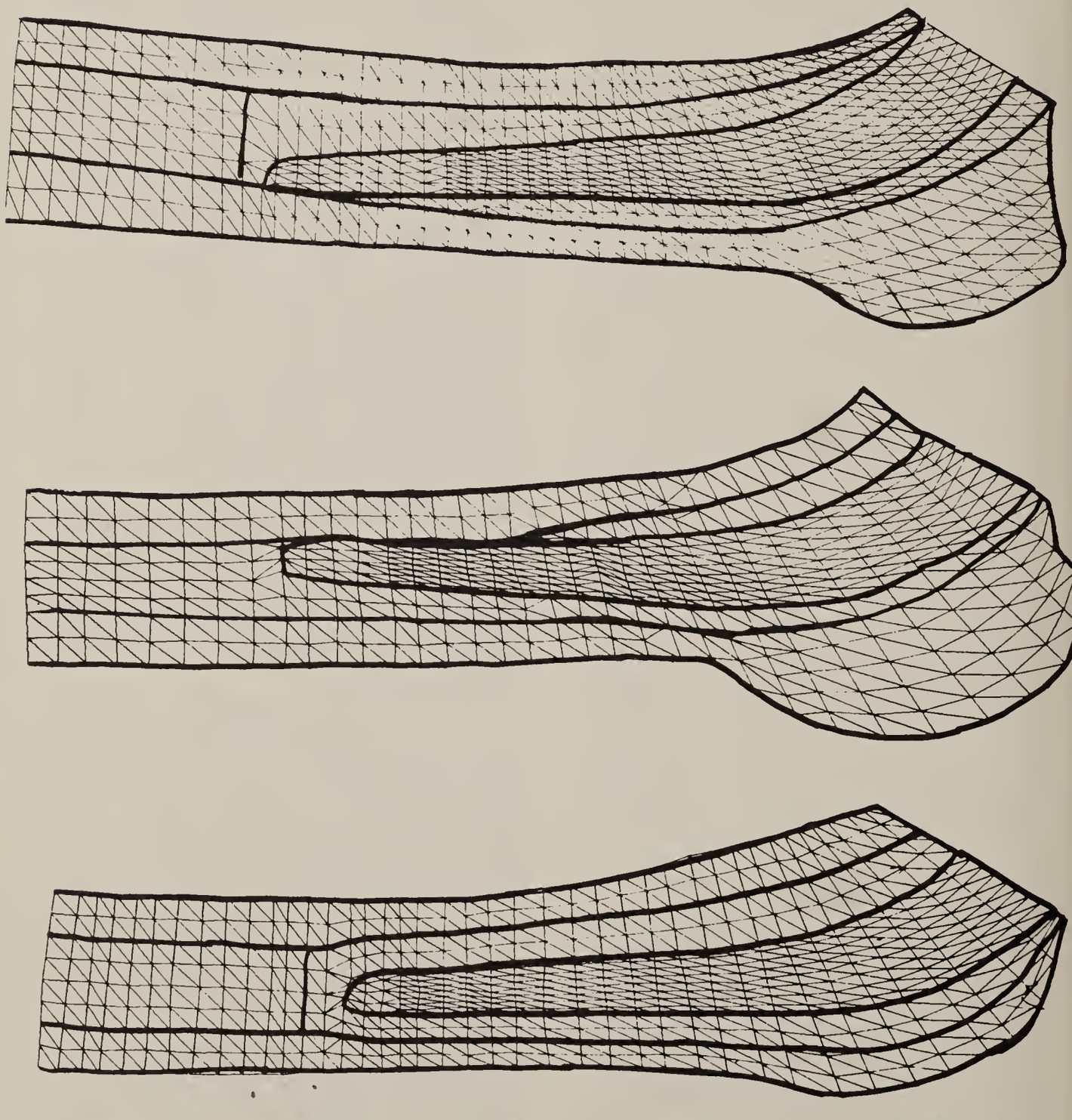


Fig. 1—Computer generated plot of a Charnley stem in varus, valgus and neutral positions.

Standard surgical techniques were used to implant the instrumented prostheses into wet, embalmed cadaver femurs. The femurs were kept wet up to the time data were recorded. The first prosthesis was implanted in an extreme valgus orientation and the second in an extreme varus. Radiographs of both implanted femoral stems are shown in Fig. 5. After the cement was allowed to set, the prosthesis was loaded statically. Loads of 100 Newtons were applied to the femoral head at 0°, 20° and 45°, with respect to the longitudinal axis of the femur. The resulting strains were measured with a Tektronix 3C66 Strain Gage Amplifier. Stresses were estimated by multiplying the measured strains by the elastic modulus of 316L stainless steel, and such stresses were then compared with the computer predictions for the varus and valgus stem orientations.

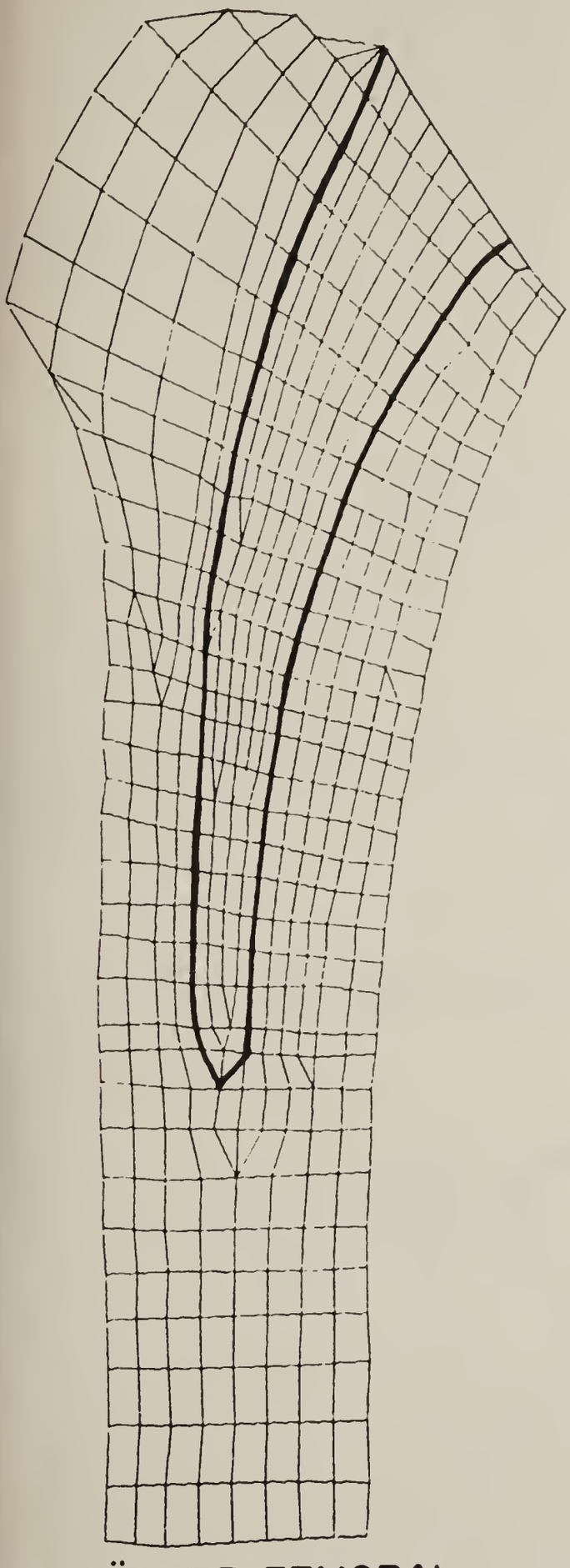


Fig. 2—Computer generated plot of Mueller stem in neutral orientation.

RESULTS AND DISCUSSION

Analytical Parameter Studies: Femoral Stem Stress Distribution

Complete stress distribution throughout the stem, bone and cement was computed by the model for each case. However, only the stress distributions in the femoral stem will be reported since this study was restricted to these factors. Shown in Fig. 6 is a typical distribution of longitudinal stresses occurring along the lateral and medial edges of the femoral stem resulting from a load of 1 Newton applied at 0°. Tensile stresses are distributed along the lateral edge, and compressive stresses along the medial edge. The stresses reach a peak magnitude at mid-stem and decrease to approximately 0 at the distal tip. Note that the region of maximum stress occurs in approximately the middle third of the stem, the region where fractures are reported clinically. Similar stress distributions to that shown in Fig. 6, were found for every stem configuration studied here, although the magnitudes of the stresses were quite different. The stress distribution shown in Fig. 6 is indicative of a bending deformation. Although the stem sustains both bending and compressive stresses, the bending stresses appear to be dominant.

Cyclical Angular Change in Load Direction—Fatigue

A noted above, the model predicts maximum tensile stresses in the region of the stem where most fractures occur. In addition to maximum tensile stresses, the range of stress fluctuations also influences fatigue life or number of cycles to failure. A standard measure of stress range is the *R* value defined as the ratio of minimum tensile stress to maximum tensile stress. As the minimum tensile stress becomes

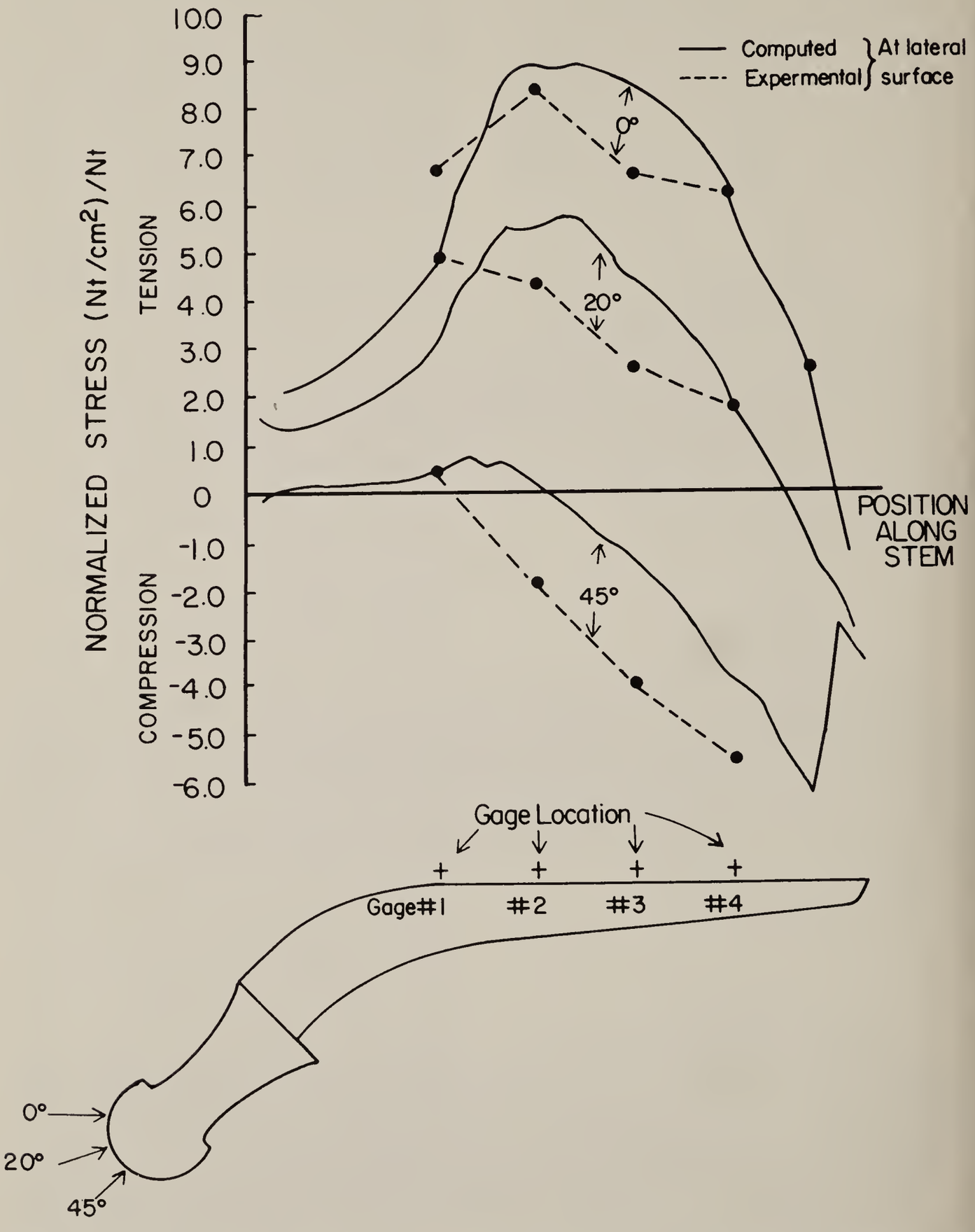


Fig. 3—A comparison of experimental and computed distributions of longitudinal stresses on the lateral surface of the femoral stem in a varus position. Stresses are normalized for a 1 Newton force. Arrows indicate direction of applied force.

compressive, the R value will become negative. As R approaches -1, the number of cycles to fatigue failure will decrease, and thus, the possibility of fatigue fracture increases. Stress fluctuations in the stem are effected by changes in the direction of load application on the femoral head. To study these effects, stresses were computed for loads applied at 0°, 20° and 45° relative to the shaft of the femur. Results are shown in Fig. 3. As expected, the highest tensile stresses resulted from a load applied at 0°. As the load approaches an angle of 45°, the stresses along the lateral surface become compressive. The stem in a varus orientation was the worst case observed; the stress fluctuation produced an R value of -0.89. In all cases studied, the R value was negative. Thus, the possibility of stress cycling due to change in load direction which yields negative R values should be accounted for in

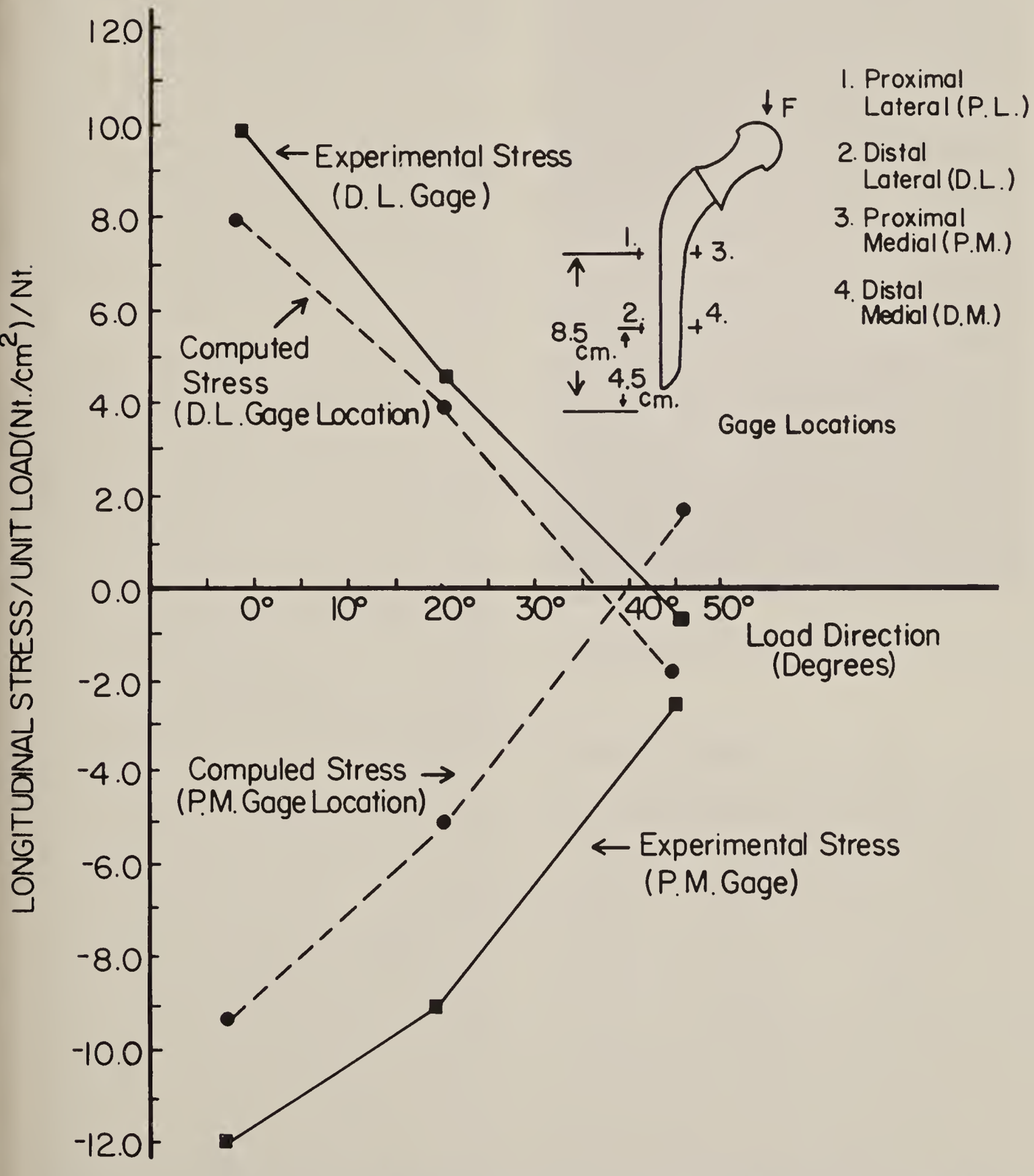


Fig. 4—A comparison of experimental and computed longitudinal stresses, at two strain gauge locations for a stem in a valgus position.

Fig. 5—Radiographs of implanted instrumented femoral stems in valgus and varus orientations.

defining maximum allowable stresses for femoral stem implant materials. For example, consider the relation between maximum allowable stress and the range of stress fluctuations for Co-Cr-Mo prosthesis alloy as reported by Miller.[16] Applying their data to the case described above ($R = -0.89$) the maximum allowable stress would be 17,700 Nt/cm^2 (25,000 psi), for 5 x 10^7 cycle lifetime, or approximately 30 years of service. The peak tensile stress shown in Fig. 4 is 9 Nt/cm^2 per Nt of applied load. Thus, a load of only 1970 Nt, or approximately 2.5 times body weight for a 700 Nt subject, would exceed the allowable stress.

Stem Orientation

Distribution of tensile stresses along the lateral surface of a Charnley stem in an extreme varus, neutral and extreme valgus orientation are shown in Fig. 7. These stress distributions were computed for a force applied at an angle of 0° relative to the shaft of the femur; the differences in magnitudes and distribution of the stresses are a result of the differences in stem orientations. The stem in a varus position sustained the highest peak stresses of the three orientations, approximately 9 Nt/cm^2 per Nt of applied load. The stem in a varus position also sustained higher stresses over a larger region of the stem length; the peak stresses sustained by the stem in valgus and neutral orientations were approximately the same magnitude, 7 Nt/cm^2 per Nt of applied load, though the stem in a neutral position sustained higher stresses over a larger region than the valgus stem.

There appear to be two factors which affect the level of stresses sustained by the stem in different orientations. The first is the moment of the load. For example, the applied force will generate larger bending moments for stem in a varus position because of a longer moment arm and thus, the stresses generated will also be larger. The support of the stem is another factor that will affect the stress levels in different orientations. If the stem is orientated differently, it will contact the cortex of the femur at different points. Cortical bone is at least an order of magnitude stiffer than bone cement, and thus, the stress distributions in the stem are affected by where the stem contacts the cortex. For example, the stem in a valgus orientation was positioned in the recommended position with lateral cortical support and medial cortical contact at the distal tip as well as proximal medial support at the calcar. When the proximal medial support was removed, the peak stresses increased to the same magnitude as those sustained by the stem in a varus orientation. Therefore, when considering stem orientation, proper support should not be neglected to reduce the moment of the applied load.

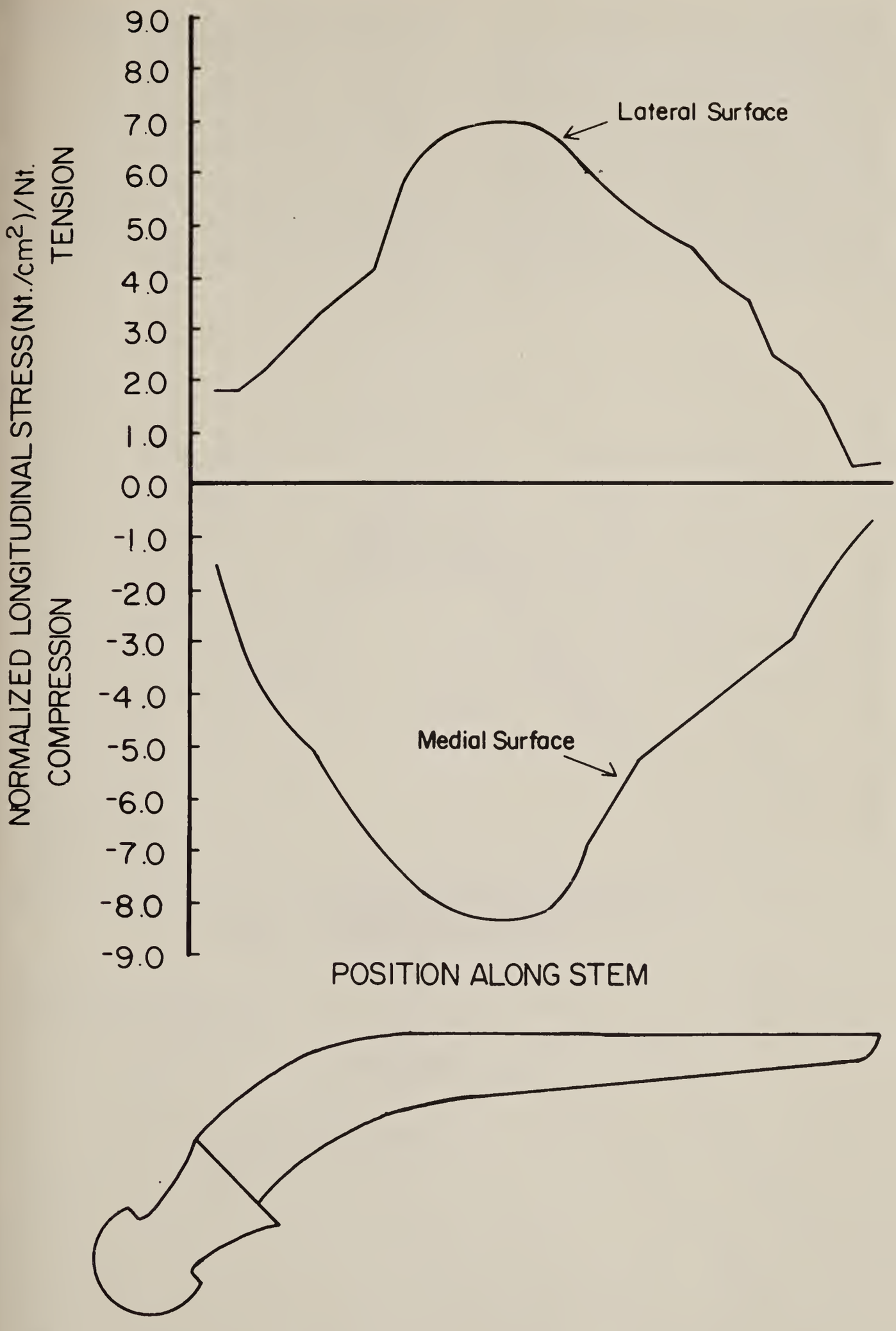


Fig. 6—A typical distribution of longitudinal stresses along the lateral and medial surfaces of a femoral stem. Results are for a Charnley stem in a neutral orientation with a force of 1 Newton applied at an angle of 0 degrees.

The Effects of an Incomplete Cement Envelope

The influence of the partial breakdown of the cement or an initially incomplete cement envelope was simulated by removing elements immediately adjacent to the stem along the proximal lateral and proximal medial quarters as shown in Fig. 8. Shown here are tensile stresses along the lateral surface for a Charnley stem in a neutral orientation. For comparison, we also show the stem stress distribution for a neutral position with a complete cement envelope. The partially cemented stem had a peak tensile stress of 9 Nt/cm² per Newton of applied load. This represented an increase of 20 percent from a completely cemented stem. It should be noted that the partially cemented stem was still supported in the region of the calcar and

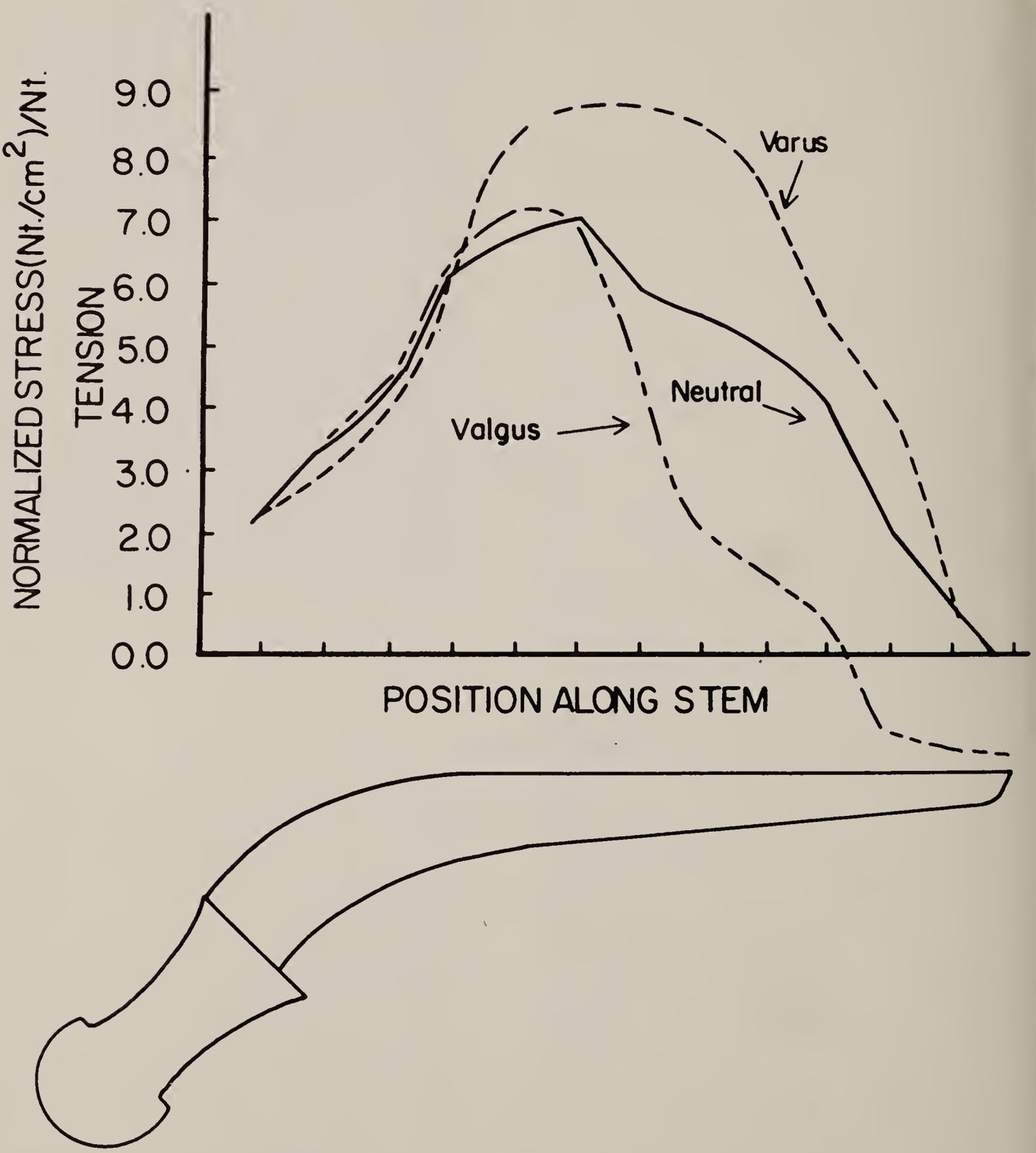


Fig. 7—The variation, due to stem orientation, of tensile stresses along the lateral surface of a Charnley femoral stem. Stresses produced by a 1 Newton force applied at an angle of 0 degrees.

the effects of incomplete calcar support were examined separately.

Charnley-Mueller Stem Design Comparison

A comparison of the stresses occurring in the Charnley and Mueller stem models for a neutral orientation is shown in Fig. 9. These stresses resulted from a load applied at 0°. The maximum tensile stresses for the Mueller stem are approximately 10 percent higher than those for the Charnley and occur more distally than peak tensile stresses for the Charnley stem. This result is consistent with the clinical findings cited in the introduction, which indicate that Charnley prostheses fail more proximally than Mueller prostheses. The Mueller stem has a reduced thickness

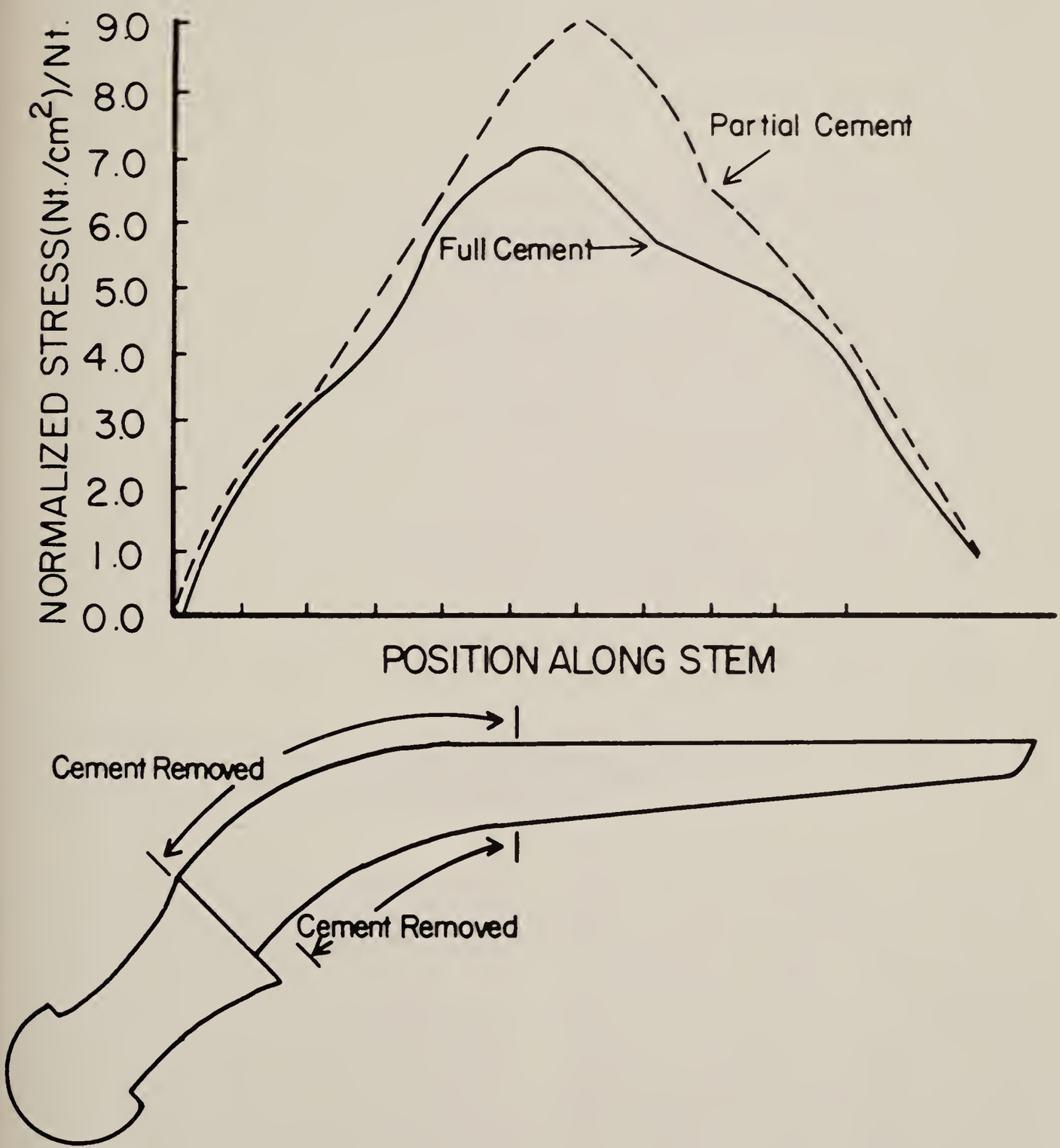


Fig. 8—An illustration of the effects of an incomplete cement envelope on the stresses along the lateral surface of a femoral stem.

at the lateral surface, so the tensile stresses there are higher than for the Charnley prosthesis. Stresses could be reduced further by increasing the cross-section at the lateral and medial surfaces. Since failures seem to occur because of high tensile stresses which result from bending deformation, it seems that a stem designed to reduce these stresses would encounter less possibilities for failure.

Calcar Support

The proximal medial support of the stem in the calcar region was assigned the elastic properties of cement, cortical bone and trabecular bone. These conditions were simulated by adjusting the Young's modulus of the elements in the calcar region for a depth of approximately 1 cm below the stem. The effect of these support conditions on the magnitude and distribution of stresses in the femoral stem is shown in Fig. 10.

For an applied load of 1 Nt the peak tensile stresses are 4.8 Nt/cm^2 for a stem supported by cortical bone and 7.6 Nt/cm^2, for a stem supported by trabecular bone. Thus, the stresses in a stem resting

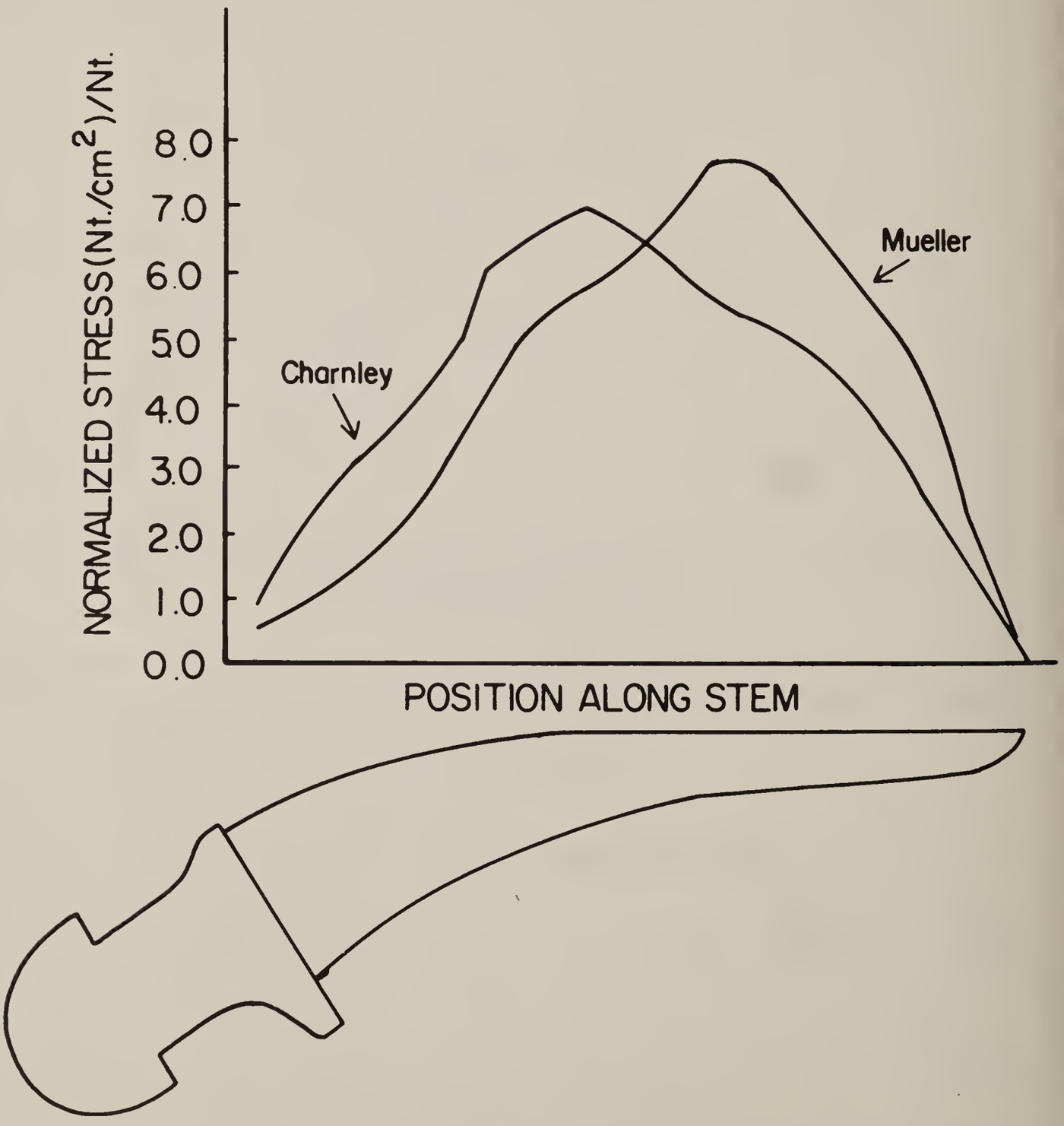


Fig. 9—A comparison of tensile stresses along the lateral surface of Charnley and Mueller prostheses. Stresses were computed for a 1 Newton load applied at an angle of 0 degrees.

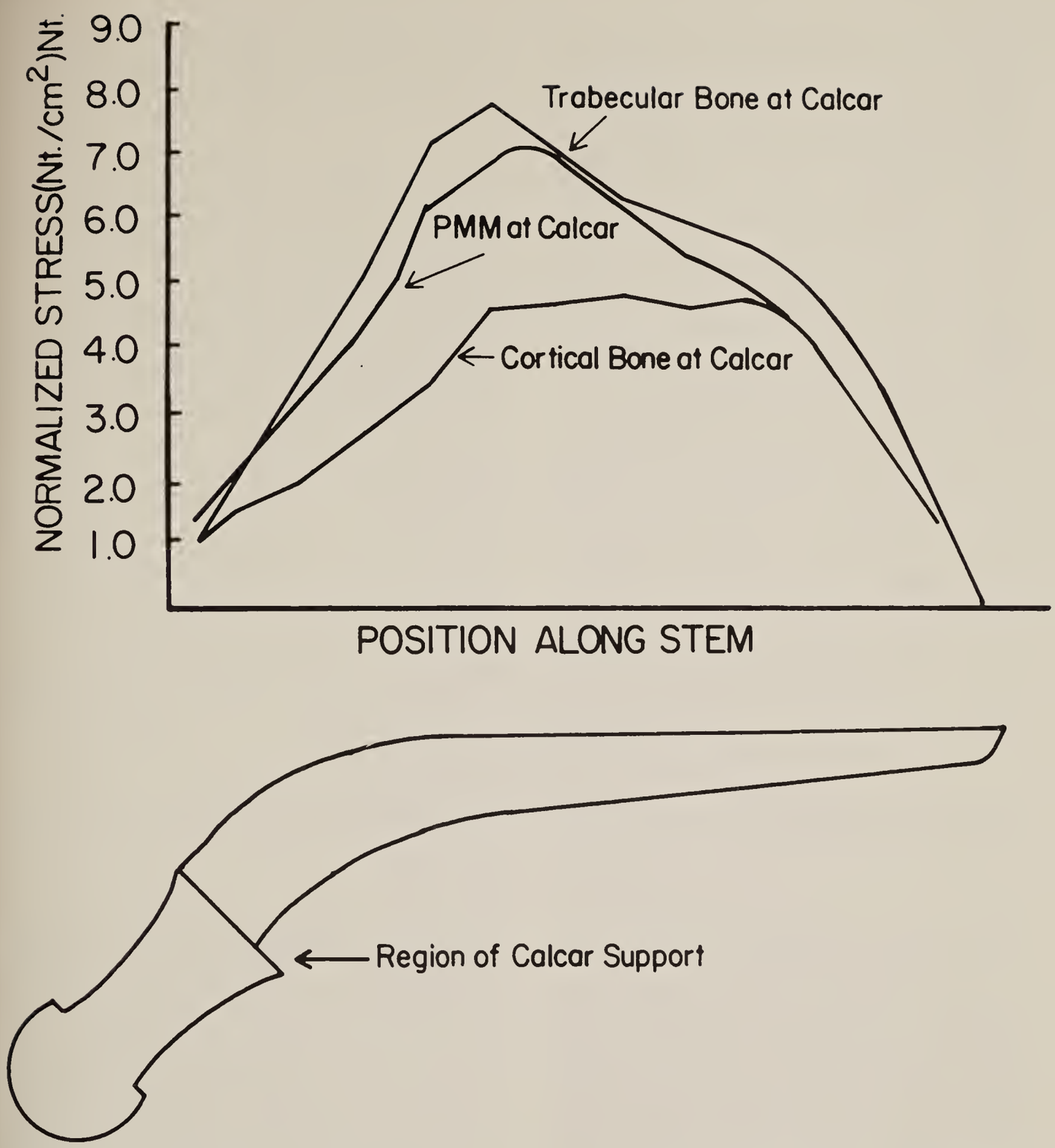


Fig. 10—An illustration of the change in the stress distribution along the lateral surface of the femoral stem resulting from various materials supporting the stem at the calcar.

on trabecular bone will be almost 60 percent higher than in one firmly positioned in cortical bone at the calcar. The stem supported by cement at the calcar had a peak stress of 7.0 Nt/cm², approximately 45 percent higher than the stresses in the stem supported by cortical bone. These results indicate that a stem resting on cortical bone at the calcar will sustain significantly lower stresses than one that is resting on a softer support. Thus, the stem support at the calcar was an important factor in maintaining low stresses in the stem. To clarify the importance of the calcar support a simple model based on engineering beam theory was developed. In the model, the stem is considered a tapered beam, simply supported at the distal tip, and the neck is assumed rigid. The calcar stiffness is idealized as a spring. As can be seen from Fig. 11, the peak stress in the stem varies inversely with the stiffness of the calcar.

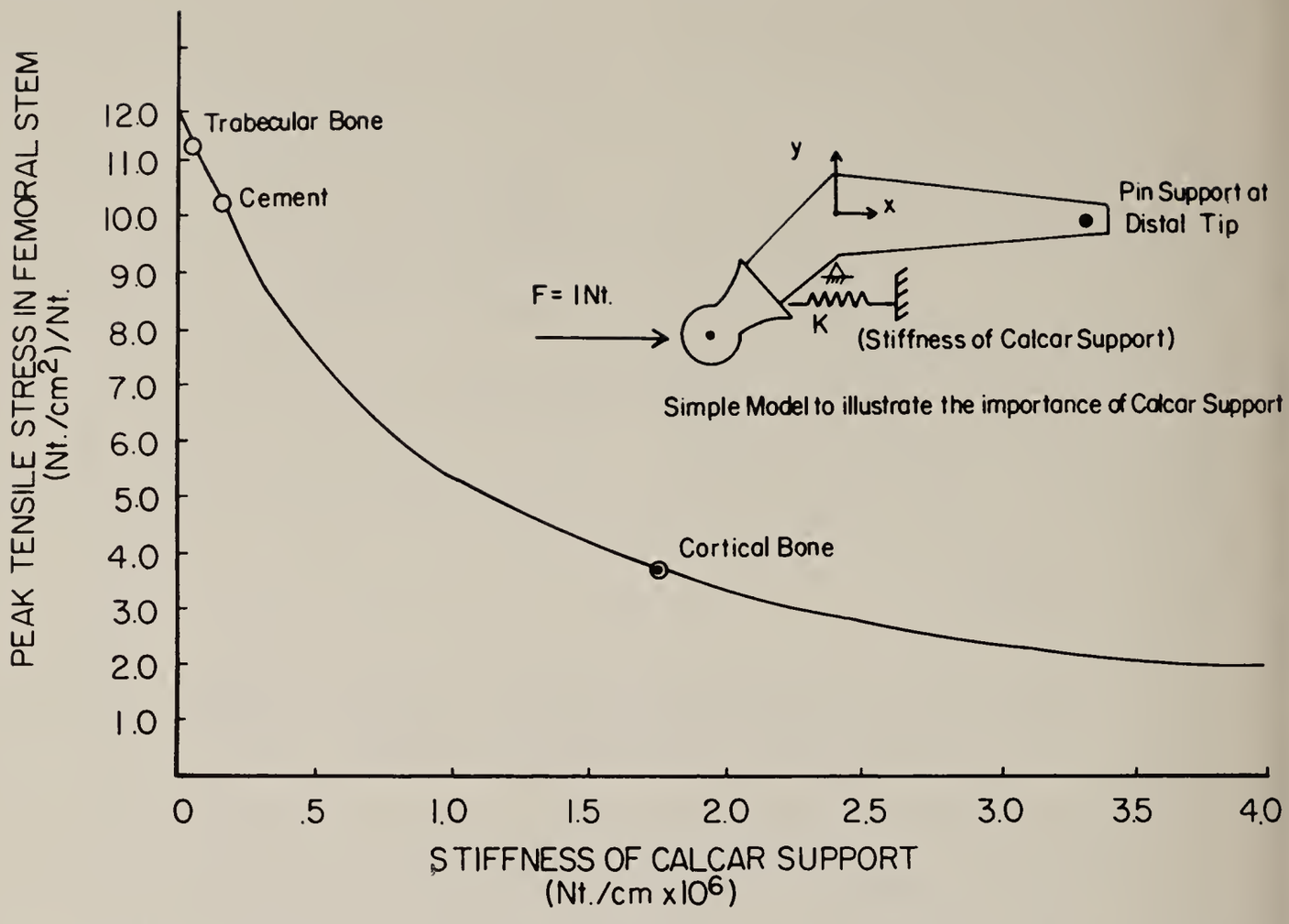


Fig. 11—An illustration of the relationship between peak tensile stress in the stem and the stiffness of the calcar support.

Comparison of Experimental and Analytical Results

Stresses measured with the first instrumented prosthesis are compared to the model predictions for a valgus Charnley stem in Fig. 4. The longitudinal stresses measured at the distal lateral and proximal medial gauges, and the predicted stresses are shown for loads applied at 0°, 20° and 45°. The stresses at the other gauge locations are shown in Table III. Both the experimental and computed stresses change in the same manner in response to changing load direction, and the distribution of stresses was consistent. However, the magnitude of the stresses found experimentally for the valgus stem were larger than those predicted by the model. In retrospect, a possible explanation for the higher experimental stresses could be inadequate support of the stem at the calcar. The predicted results were based on a stem firmly resting on the calcar; analytical results previously described indicate that inadequate stem support at the calcar will increase the stresses in the

TABLE III
A COMPARISON OF EXPERIMENTAL AND COMPUTED LONGITUDINAL STRESSES FOR A VALGUS SYSTEM

	*Longitudinal Stresses (Nt/cm²)/Nt					
	Load Angle = 0°		Load Angle = 20°		Load Angle = 45°	
Gauge Position	Experimental	Computed	Experimental	Computed	Experimental	Computed
Distal Lateral	10.0	8.0	4.8	4.7	−1.0	−2.0
Proximal Lateral	7.4	6.2	2.2	3.0	−3.7	−2.0
Distal Medial	−12.4	−9.1	−9.0	4.8	−2.2	−2.0
Proximal Medial	—	−7.2	—	−4.0	—	−1.8

*Tensile stresses are indicated as positive

stem substantially. Unfortunately, the first prosthesis was removed from the femur before the calcar support could be examined.

A second prosthesis was implanted in a varus orientation. The support at the calcar was carefully examined and it was found that the stem was supported by cement in the region of the calcar. These results are compared with the model predictions for stems supported by cement and not cortical bone, in the region of the calcar in Fig. 3. The stress distributions predicted by the model and the measured stresses at the four gauge locations along the lateral surface were compared for loads applied at 0°, 20° and 45°. Again, the behaviour of stresses for different directions of applied force for experimental and computed results were consistent. However, the model predicted somewhat higher tensile stresses than those found experimentally.

It should be noted again that the computer results are based on a two-dimensional model. The simulated femoral stem is supported only along its lateral and medial surfaces. For certain orientations and positions, the stem may contact only cement or both cement and bone. The model stems thus derive no support from cement or bone lying out of the plane of the prosthesis. It would seem reasonable to expect the computed stresses to be higher than those found experimentally, since the actual implant is completely surrounded by cement and bone. However, the computed and experimental stresses agree well within the range of experimental error. On the basis of these findings, it appears that support at the boundaries along the lateral and medial surface is dominant, and that any support derived from cement and bone not in the plane of the prosthesis probably will play a secondary role for the types of loads assumed in this study.

SUMMARY AND CONCLUSIONS

A two-dimensional finite element stress analysis has been used to study the effects of several factors on stress levels in the femoral stem of total hip prostheses. *In vitro* experiments, using femoral stems instrumented with strain gauges, were used to corroborate the predictions of these analyses. Although neither the analytical nor the experimental results provide a direct measure of *in vivo* stresses, we have provided evidence of the validity of the results. In summarizing this evidence, it is well to bear in mind that there are essentially two basic steps in the development of our analysis: first the concept of the implanted femur as a passive system with material properties based on cadaver studies, and secondly, the simplification of the three-dimensional geometry of the implanted femur.

The first assumption, that *in vivo* stresses are represented by these studies, hinges mainly on the fact that the conclusions of this study are based on comparisons which are not sensitive to the material parameters. Thus, all of the findings reported here should remain valid regardless of whether certain bone or cement constants are changed 20 percent or 30 percent. As to the second simplification, analytical justifications of the model are given in Andriacchi,[8] but in any case the reasonable agreement between analytical results seemed sufficient to confirm the appropriateness of this model for the study of femoral stresses. Finally, all of the findings are consistent with clinical experience. For example, the regions of high tensile stress are the regions where stem fractures are observed clinically.

The following observations were made regarding the stress levels in the femoral stem:

1. Bending deformation, which produces tensile stresses along the lateral surface and compressive stresses along the medial surface, is predominant in most situations. The tensile stresses are maximum in the middle third of the stem and diminish to zero at the distal tip. A simple engineering model, illustrated in Fig. 11, can be used to qualitatively represent the behaviour of the stem.

2. Variations in the direction of the applied load from 0° to 45° result in stress

reversals in the stem. In the worst case, a varus position, stem stress cycling yielded an R value of -0.89. These results indicate that design criteria for stem implants should take into account the possibility of nearly reverse bending; otherwise fatigue failure is a distinct possibility, despite low maximum stress levels.

3. One factor that affects peak tensile stresses is the orientation of the stem. Analytical results show that a varus stem sustains 20 percent higher stresses than stems in neutral or valgus orientations. For any given orientation, the maximum stress depends on the moment of the load and the support of the stem.

4. The support of the stem by the cement envelope, especially in the region of the calcar, appears to be the most important factor in determining stress levels in the femoral stem. It was shown that a stem resting on trabecular bone at the calcar will have maximum stresses 60 percent higher than a stem resting directly on cortical bone.

To summarize, the results of this study indicate that the achievement of adequate stem support is most important in preventing excessive stresses in the femoral stem. While stem orientation plays a role in reducing tensile stresses in the stem, the surgeon should not neglect the need for adequate stem support at the calcar in attempting to achieve proper orientation. These results are also pertinent in the design of new prostheses. Stem designs should incorporate some means of insuring adequate support at the calcar, throughout the life of the prosthesis. In addition, stem cross-sections should be designed to achieve maximum stiffness in the middle one-third of the stem, where the maximum tensile stresses occur, though any increase in cross-sectional dimensions must be weighed against the restrictions imposed by the surgical procedure and femur geometry.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the contributions to this research of Robert Miller, M.D., James Ogle and Allan Strongwater. This research was supported in part by Public Health Service Grant AM 16485 and Fellowship Award AM 05020-01.

REFERENCES

1. **Galante J, Rostoker W, Doyle J:** Failed femoral stems in total hip prostheses. *J Bone Joint Surg 57A:230, 1975*
2. **Martens M, Aernoudt E, De Meester P, Ducheyne P, Mulier JC, De Langh R, Kestelijn P:** Factors in the mechanical failure of the femoral component in total hip prosthesis. *Acta Orthop Scand 45:693, 1974*
3. **Charnley J:** Fatigue fracture of femoral prosthesis in total hip replacements—a clinical study. Centre for Hip Surgery, Weightington Hospital, Internal Publication No. 52, 1974
4. **Rybicki EF, Simonen FA, Weis EB:** On the mathematical analysis of stress in the human femur. *J Biomech 5:203, 1972*
5. **Belytschko T, Kulak R, Schultz A, Galante J:** Finite element stress analysis of an intervertebral disc. *J Biomech 7:277, 1974*
6. **Meek JL:** *Matrix Structural Analysis.* New York, McGraw-Hill, 1971
7. **Zienkiewicz OC:** *The Finlte Element Method in Engineering Science.* London, McGraw-Hill, 1971
8. **Andriacchi T, Hampton S, Belytschko T, Galante J:** The application of finite element method to studies of implant stresses. Unpublished data
9. **Reilly DT, Burstein AH, Frankel VH:** The elastic modules for bone. *J Biomech 7:271, 1974*
10. **Bonfield W, Datta PK:** Young's modulus of compact bone. *J Biomech 7:147, 1975*
11. **Pope MH, Outwater JO:** Mechanical properties of bone as a function of position and orientation. *J Biomech 7:61, 1974*
12. **Lautenschlager E:** Unpublished data. Northwestern University, Department of Biological Materials, Chicago, Illinois, 1974
13. **Rydell NW:** Forces acting on the femoral head-prosthesis. Thesis from the Department of Orthopaedic Surgery, University of Goteborg, Sweden, 1966
14. **Paul JP:** Load actions on the human femur in walking, and some resultant stresses. *Exp Mech, 1971*
15. **Williams JF, Svensson ML:** A force analysis of the hip joint. *Biomed Eng 3: No. 1, 365, 1968*
16. **Miller H, Rostoker W, Galante J:** A statistical treatment of fatigue on the cast Co-Cr-Mo prosthesis alloy. *J Blomed Mater Res (in press)*

COMPUTED TOMOGRAPHY OF THE HEAD

Dennis W. Boyce, M.D.
Michael S. Huckman, M.D.

ABSTRACT. Computed tomography of the head is a new, non-invasive radiologic technique which is capable of diagnosing many intracranial diseases. A review of the various lesions detectable by this modality is presented.

INTRODUCTION

Computed tomography of the head is a recently-developed, non-invasive radiologic technique that allows analysis of normal and pathological cerebral structures, without risk to the patient.[1] This technique, developed by Houndsfield,[2] employs a narrow beam of x-rays which passes through the head, and sodium iodide crystals which detect the amount of transmitted radiation. The tightly-collimated beam of x-rays and opposed detector scan the head in a transverse plane, angled approximately 25 degrees toward the feet. After each scan, the gantry on which the tube and detectors are mounted rotates one degree (Fig. 1). This is repeated 180 times in five minutes, producing 43,800 readings of x-ray density. This information is then used to reconstruct a cross-section image of the head on a 160 x 160 matrix. The data are displayed in analog form as a cathode-ray tube reproduction in which the brightness of each point is proportional to the average radiodensity of the tissue in the corresponding section of brain. Polaroid photographs of the cathode-ray tube image provide a permanent record of each examination. The

From the Department of Diagnostic Radiology, Section of Neuroradiology, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois

Dennis W. Boyce, M.D., Fellow in Neuroradiology, Presbyterian-St. Luke's Hospital; Instructor in Radiology, Rush Medical College

Michael S. Huckman, M.D., Director, Section of Neuroradiology, Presbyterian-St. Luke's Hospital; Associate Professor in Radiology, Rush Medical College

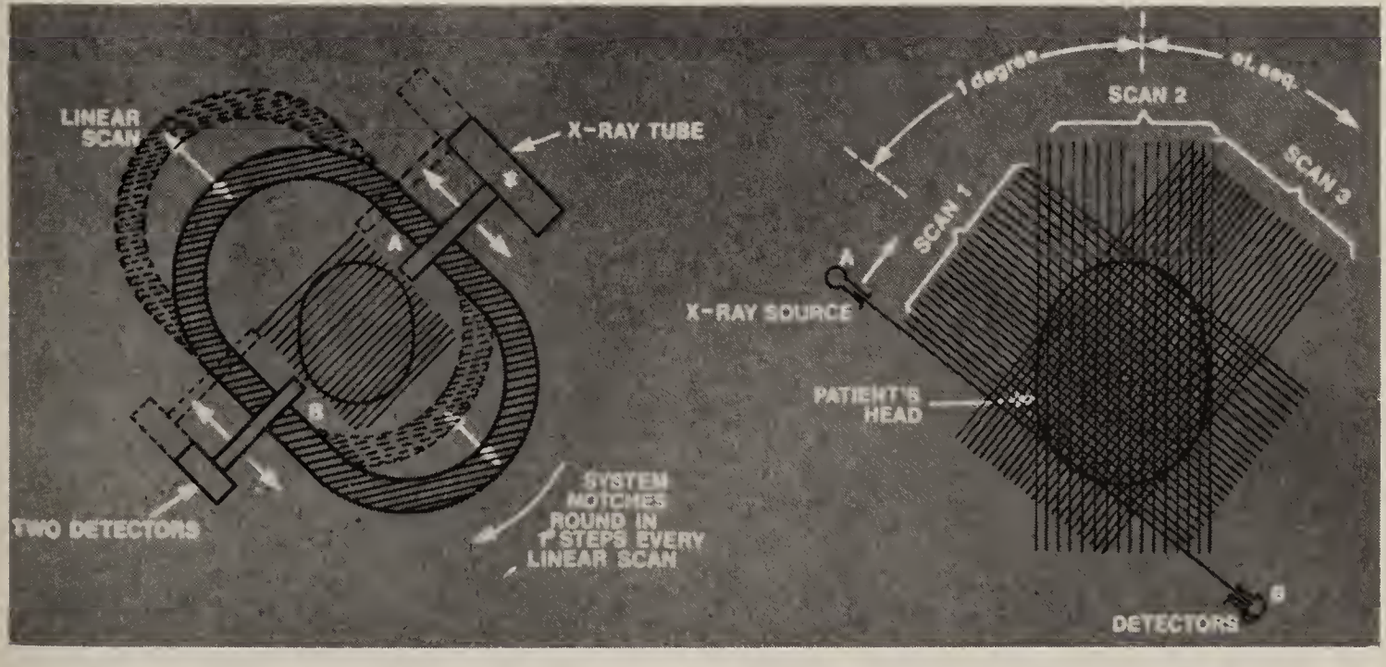


Fig. 1—Schematic of scanner gantry motion *(Courtesy of EMI, Ltd., Middlesex, England).*

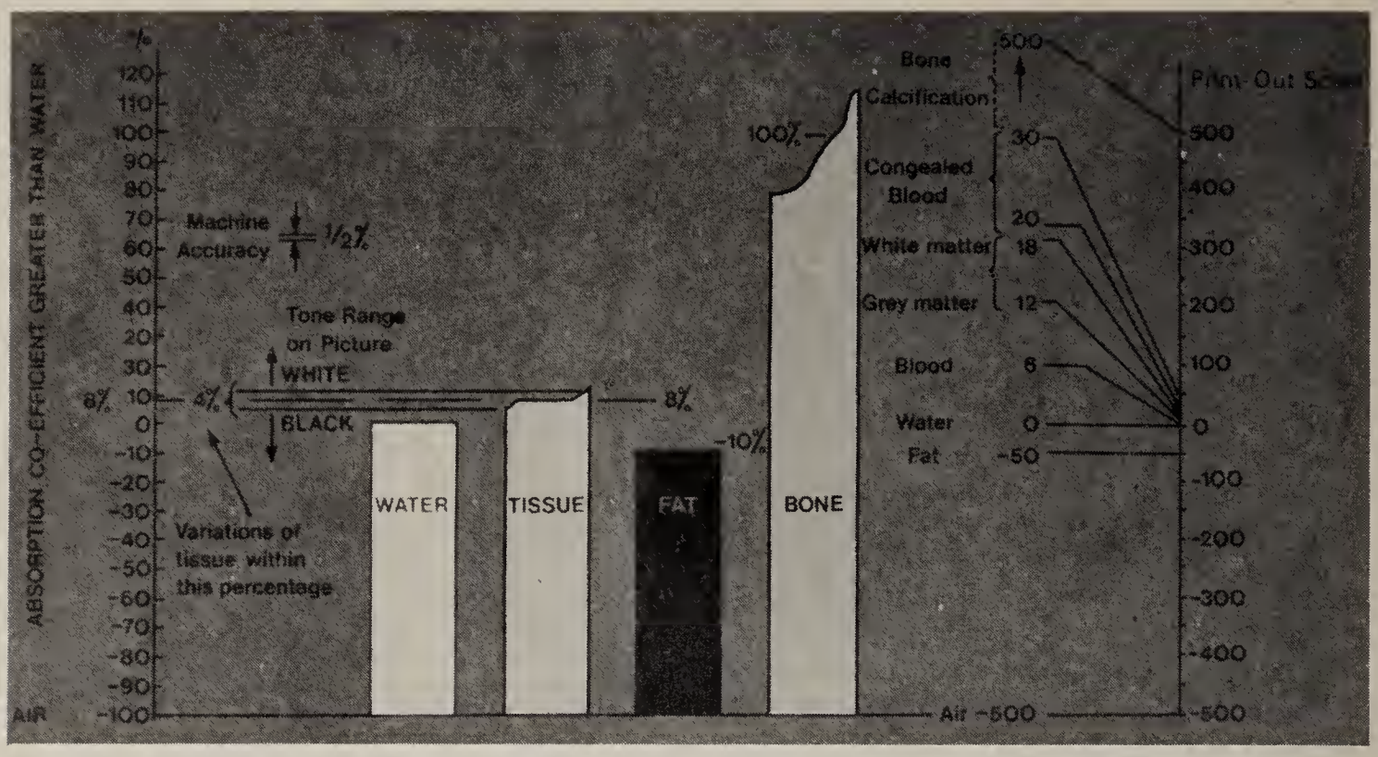


Fig. 2—Scale of absorption values, in percentages relative to water (zero). *(EMI, Ltd.)*

data can be stored on magnetic tape, so that density measurements, as well as the image, can be retrieved. Arbitrarily, a scale of densities is used where air is −500 units, water is 0, and +500 is dense bone (Fig. 2). Abnormalities, as well as normal vascular structures, are enhanced following the intravenous infusion of iodinated contrast material (250 ml of 25 percent sodium diatrizoate). Neovascularity within or about a tumor will increase in density with intravascular contrast material. Contrast may cross the blood-brain barrier in certain pathologic states to produce the enhancement of lesions.[3]

Approximately 50 percent of patients receive contrast infusion because of suspicion of a primary or metastatic tumor, arteriovenous malformation, subdural hematoma or abscess.

NORMAL SCAN

To study most of the brain on the scan, we position patient's head within the scanning device so that the plane of the slice is approximately 25 degrees toward the feet, to insure inclusion of the posterior fossa on the scan (Fig. 3). In most patients, eight tomographic slices (each 13 mm thick) will include most of the brain (Fig. 4). Bone and calcification appear white on the cathode ray tube; air and cerebropsinal fluid are black; and normal brain has varying shades of gray. By utilizing the control panel, we can measure the density of various structures allowing for differentiation of various normal and pathological processes.

Calcium in the pineal gland and glomus of the choroid plexus are readily detected (white) even when they are not

Fig. 3—A patient in the scanner.

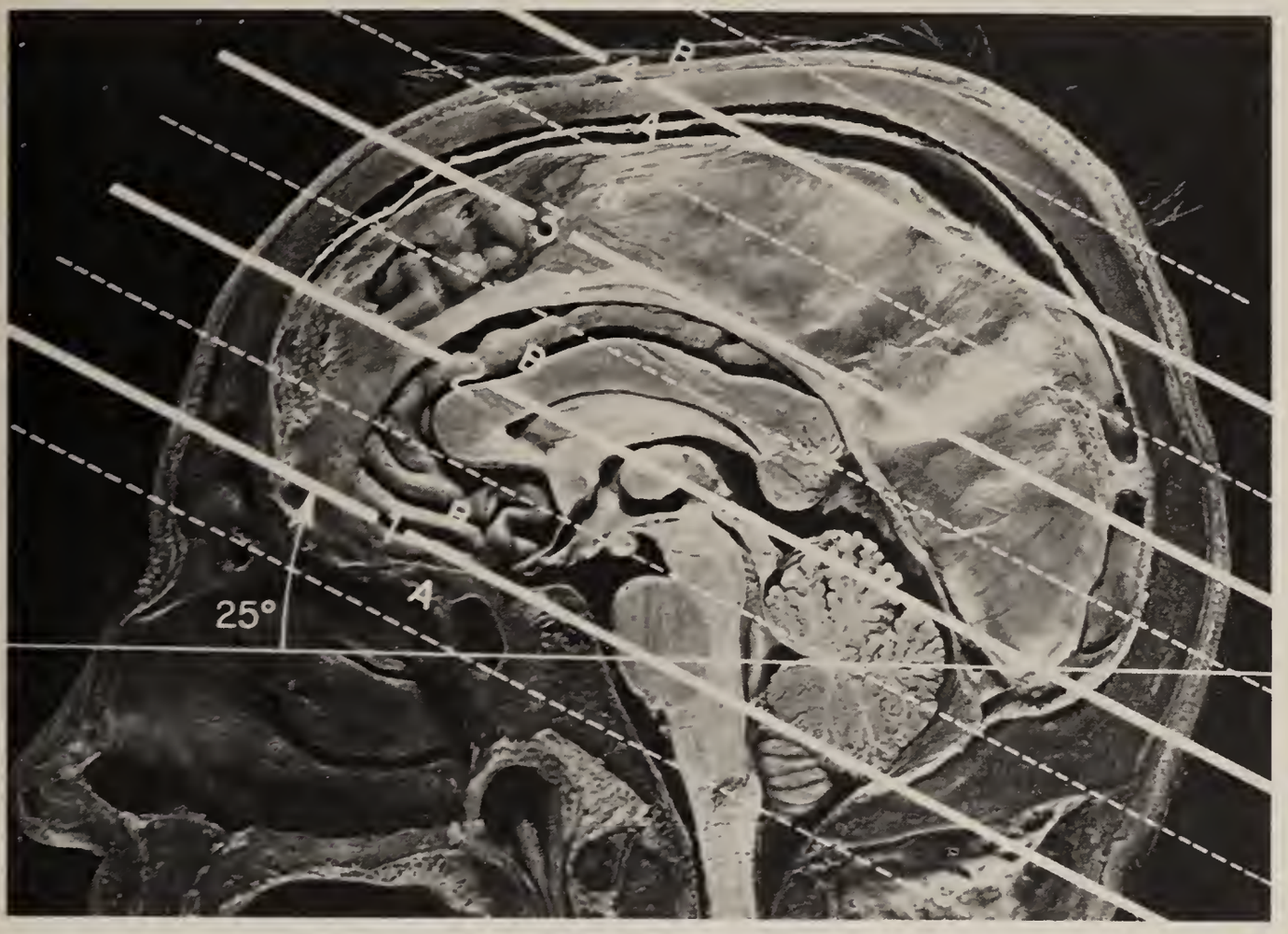


Fig. 4—Mid-sagittal section of a cadaver with anatomical slices, 1.3 cm thick, on either side of the superimposed reference lines (heavy white lines).

discernible on plain skull radiography. The ventricular system filled with low density (black) cerebrospinal fluid is easily seen. Subarachnoid spaces (sylvian fissures, suprasellar and quadrigeminal cisterns, and enlarged cortical sulci) are often identified (Fig. 5).

ABNORMAL SCAN

Primary Neoplasms

Primary neoplasms of the brain typically alter the parenchymal density. Usually, low-grade astrocytomas appear as a mass with irregular, frond-like contours which is less dense than normal brain. Glioblastomas are more heterogeneous with areas of increased density.[4] Edema (low density) usually surrounds the tumor. Mass effect can produce distortion of the ventricular system and shift of midline structures. If the tumor is located adjacent to the ventricular pathways, it can produce obstructive hydrocephalus. Following the infusion of contrast, neoplasms usually show a patchy, irregular increase in density. A dense, shaggy peripheral ring of density suggests neovascularity in a high-grade glioma. The central black areas represent necrotic and cystic change with the tumor (Fig. 6).

Occasionally, a neoplasm may go undetected on plain scanning. The base cut (Fig. 7, Cut 1B) does show a large sella turcica. Following infusion of contrast, the tumor increases in density (Fig. 7, Cut 7A). At surgery a chromophobe adenoma with suprasellar extension was removed.

Metastatic Neoplasms

Metastatic neoplasms change the density of the brain and often are barely discernible in the plain scan, especially if balancing lesions prevent a shift of the midline structures and demonstration of mass effect. Metastatic malignant melanoma produces dense lesions on the plain

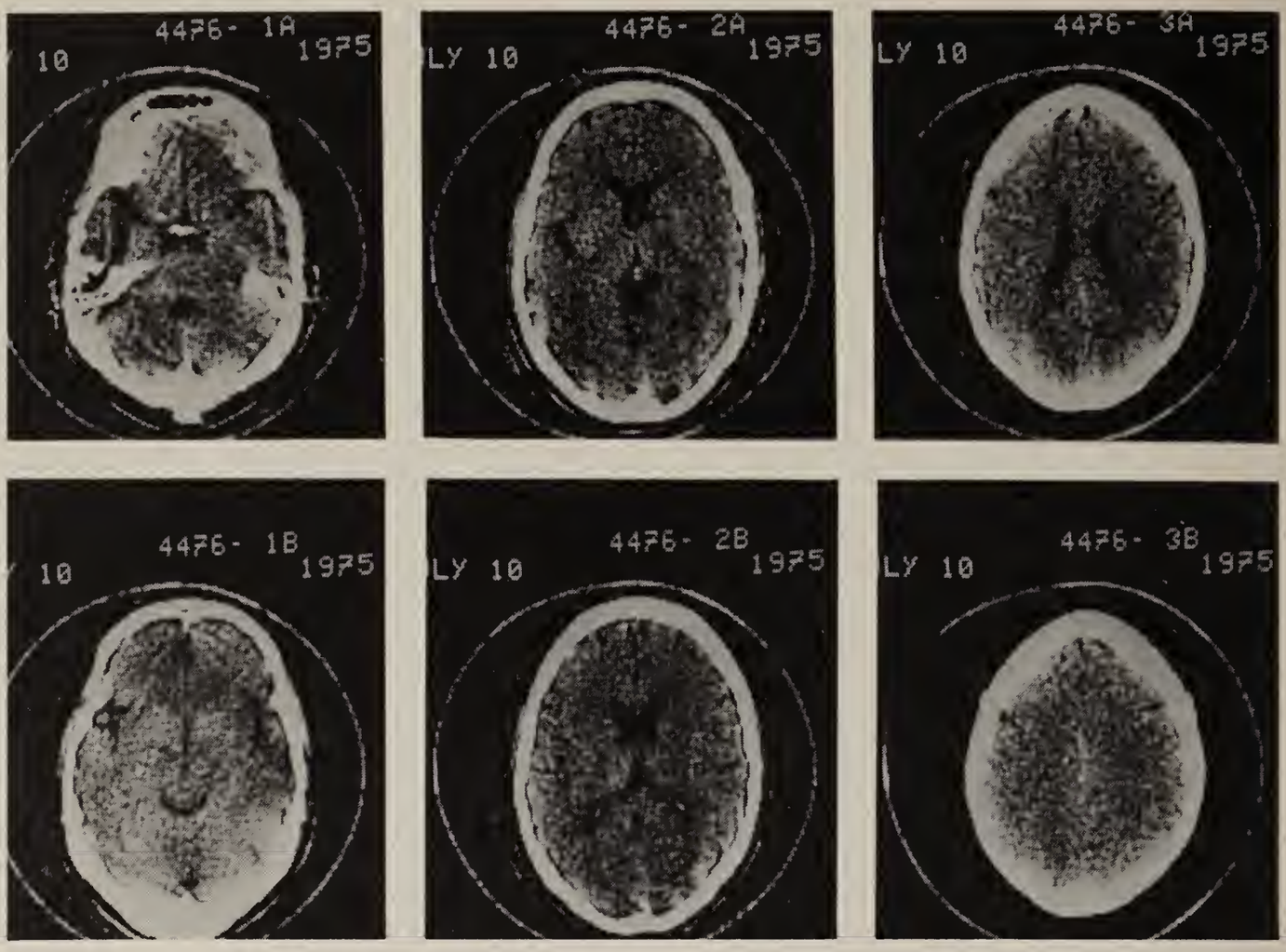
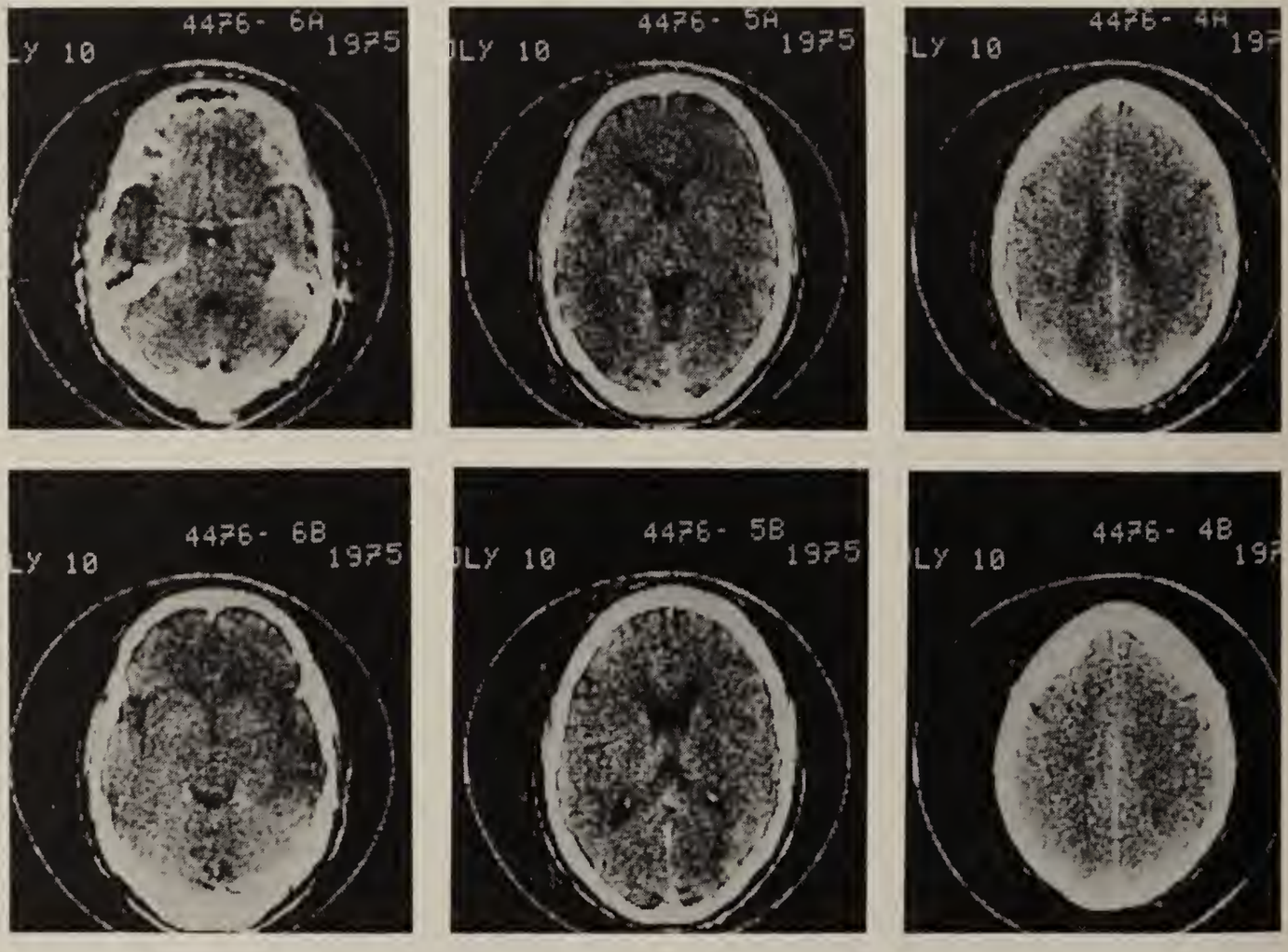


Fig. 5A—Normal computed tomogram (C.T.). The lowest "slice" is 1A; the highest is 3B. The right side of the head is on the reader's right.

Fig. 5B—Normal post-infusion C.T. The lowest "slice" is 6A; the highest 4B.



scan but, surprisingly, following contrast infusion, unsuspected lesions will appear (Fig. 8).

Hemangioma

Vascular tumors are readily detected when iodinated contrast material is in the intravascular space. A hemangioma posterior to the right eye is seen to produce asymmetry of the position of the globe in the orbit. It appears as a round density which increases in density following comtrast enhancement (Fig. 9). Likewise, arteriovenous malformations dramatically increase in density with contrast infusion.

Hemorrhage

Extravasated blood has high density (range 25 to 45 units) whereas circulating intravascular blood has low density (6 units). Soon after extravasation, the density increases markedly; the cause of this phenomenon is not yet clear. In general, hematomas are readily identified. If located near the base of the brain, the hematoma may be caused by a ruptured aneurysm. However, angiography is necessary for detailed evaluation. In Fig. 10, hemorrhage into the left basal ganglion presented on the scan with mass effect and rupture into the ventricular system. Partial resolution of the hematoma, decreased density, and mass effect were noted sixteen days later.

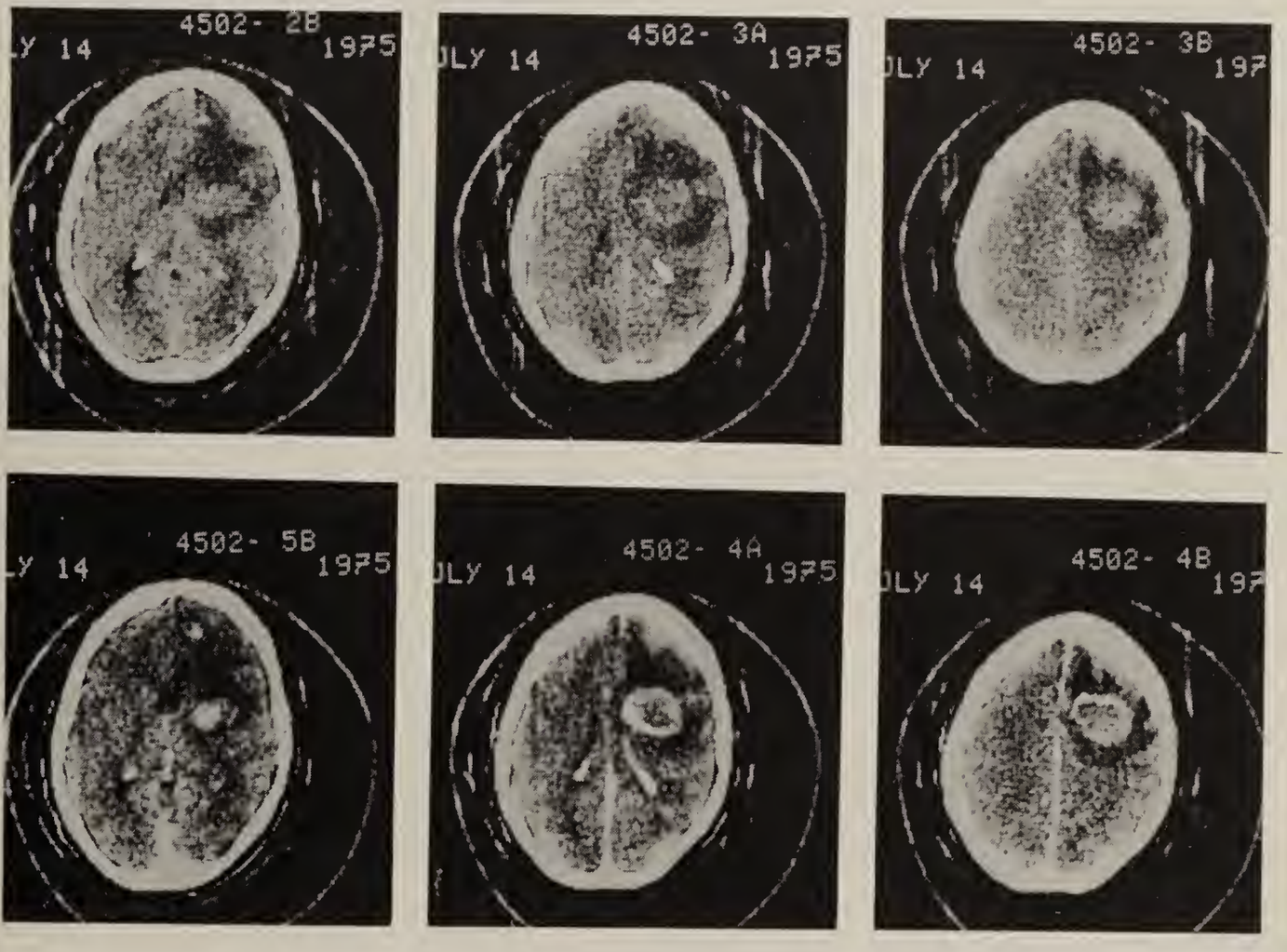
Hematoma

Extracerebral hemorrhage, subdural, and epidural hematomas, when acute, are high-density collections which dislocate the midline and compress the ventricular system. Chronic subdural hematomas have an intermediate-to-low density (if liquefaction has occurred) and can be more difficult to detect. Bilateral, chronic frontal subdural hematomas produce almost symmetric compression of the frontal horns of the lateral ventricles, but no shift of the midline (Fig. 11).

Infarction

A patient with recent infarction which has occurred within a few to 48 hours,

Fig. 6—Astrocytoma Grade III. Pre-infusion: 2B, 3A, 3B. Corresponding levels after infusion: 5B, 4A, 4B.



may have a normal scan. After that time, associated cerebral edema may present as a patchy area of decreased density and may exert mass effect. Infusion of contrast may "light up" several areas around the lesion because of "luxury perfusion"—the opening of collateral vascular pathways to perfuse the damaged brain tissue. One must be cautious not to interpret this contrast enhancement as indicative of a neoplasm. Old infarcts appear as well-defined, low density lesions (Fig. 12). When the involved tissue decreases in volume, it can produce adjacent sulcus or ventricular enlargement.

Hydrocephalus

The low density of cerebrospinal fluid permits ready measurement of ventricular size without discomfort to the patient. Production of artifactual dilation by air at pneumoencephalography is also avoided. Hydranencephaly in a newborn (failure of development of the cerebral hemis-

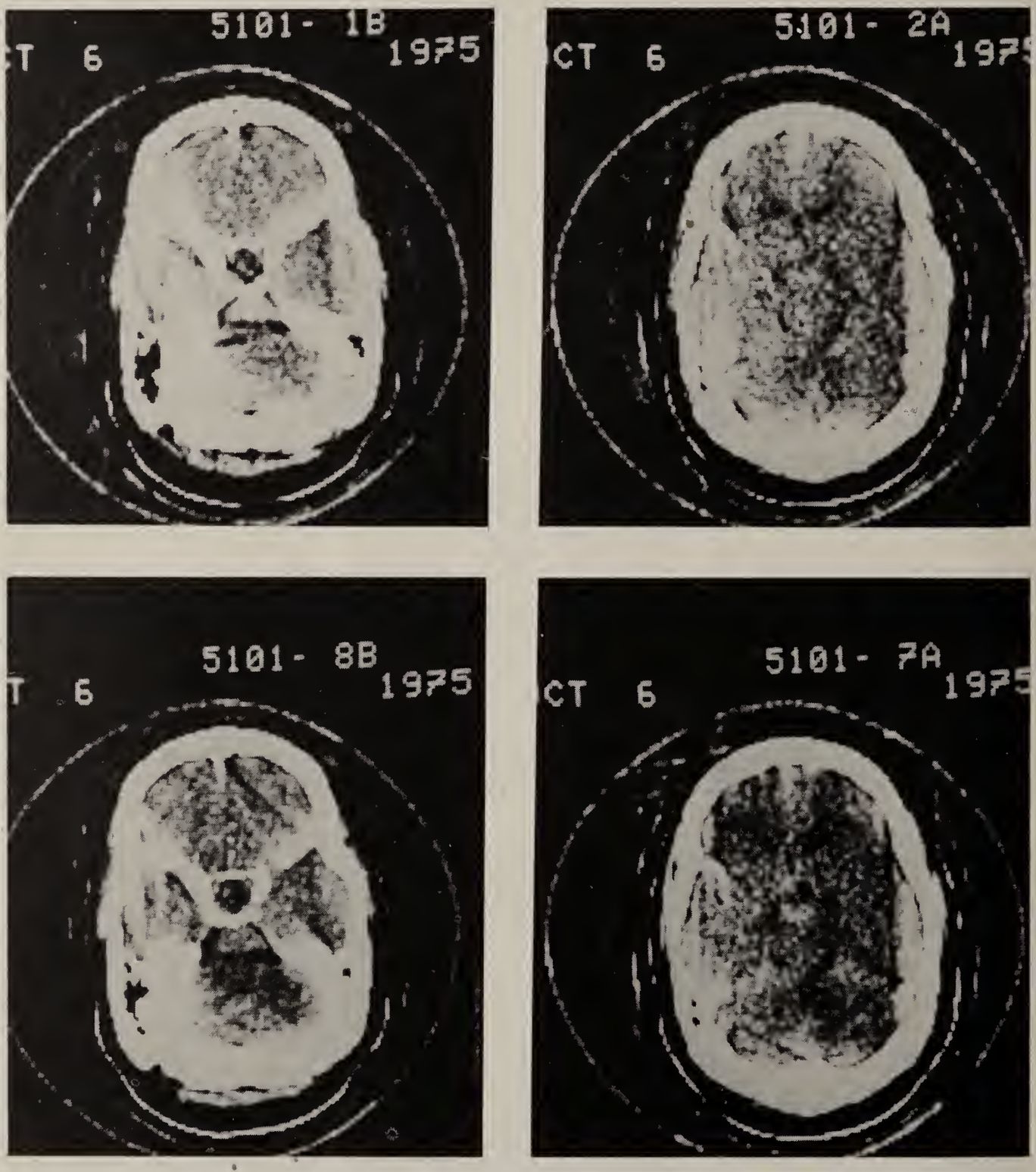


Fig. 7—Chromophobe adenoma with suprasellar extension. Pre-infusion: 1B, 2A. Post-infusion: 8B, 7A.

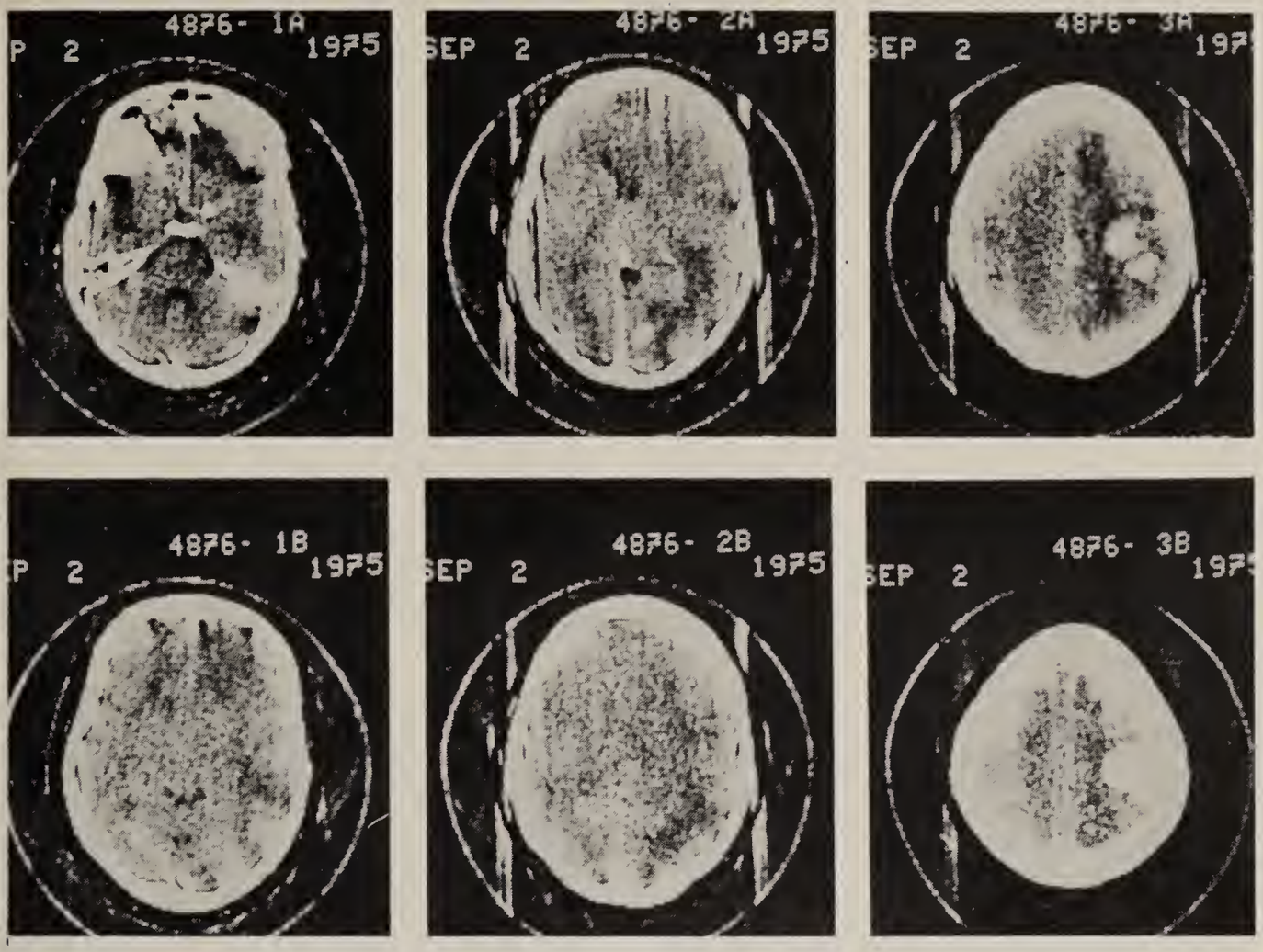
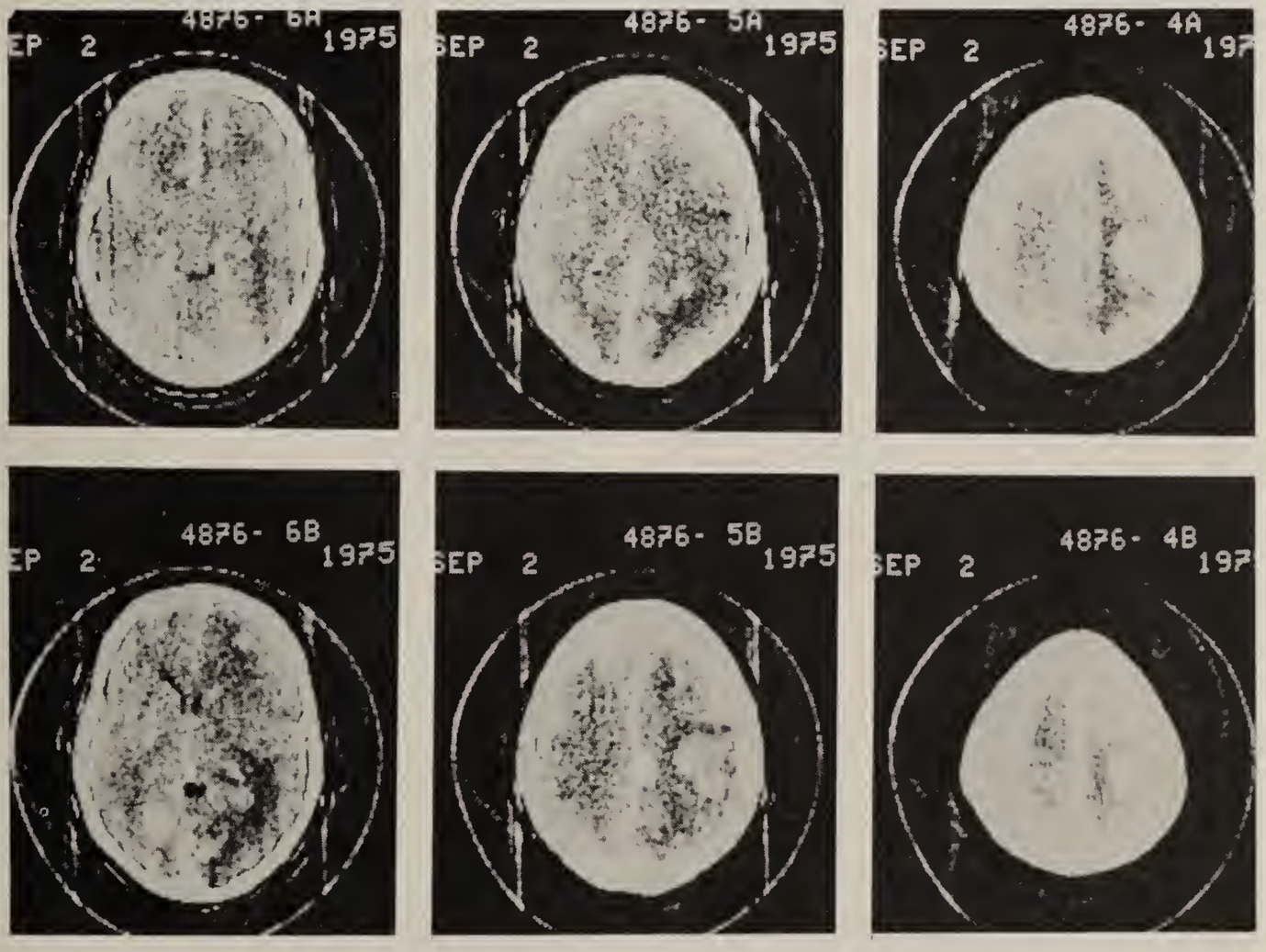


Fig. 8A—Metastatic melanoma, pre-infusion.

Fig. 8B—Metastatic melanoma, post-infusion.



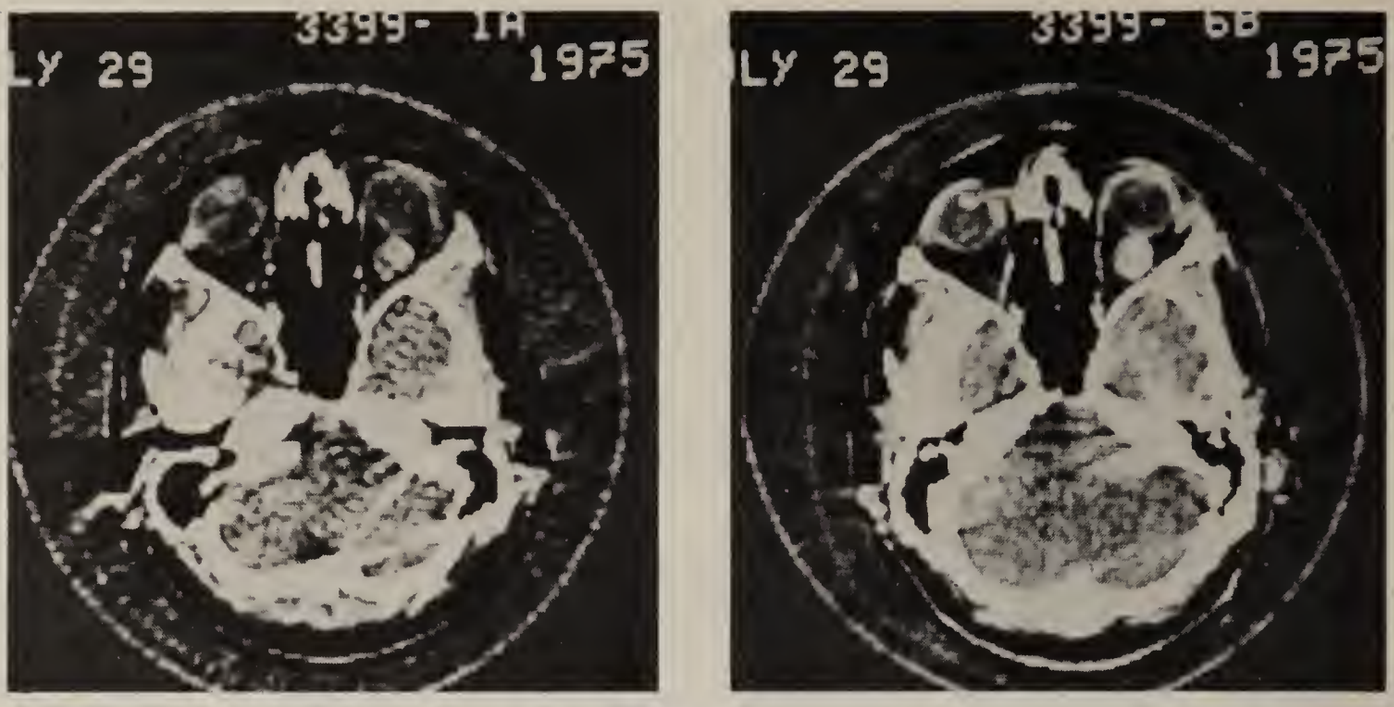


Fig. 9—Hemangioma posterior to the eye in the right orbit. 1A: pre-infusion; 6B, post-infusion.

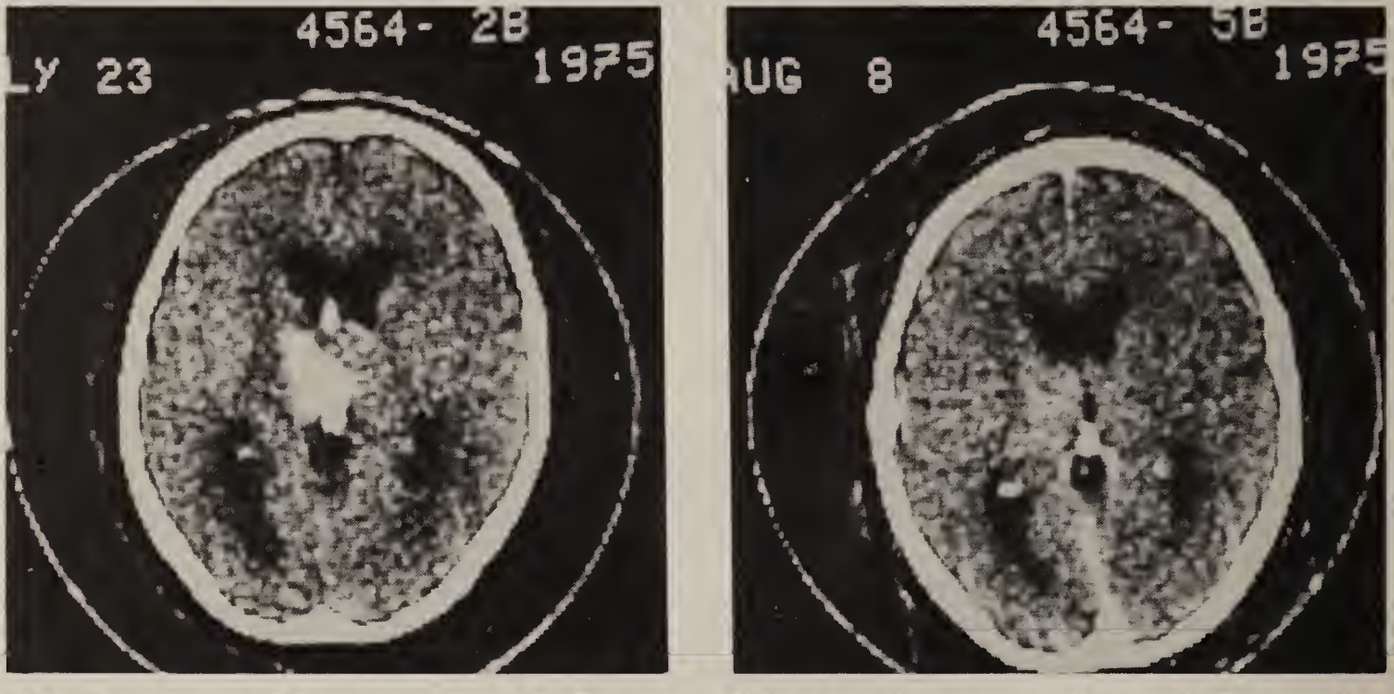


Fig. 10—Hemorrhage into left basal ganglion acutely (left scan). Sixteen days later (right scan), partial resolution has occurred.

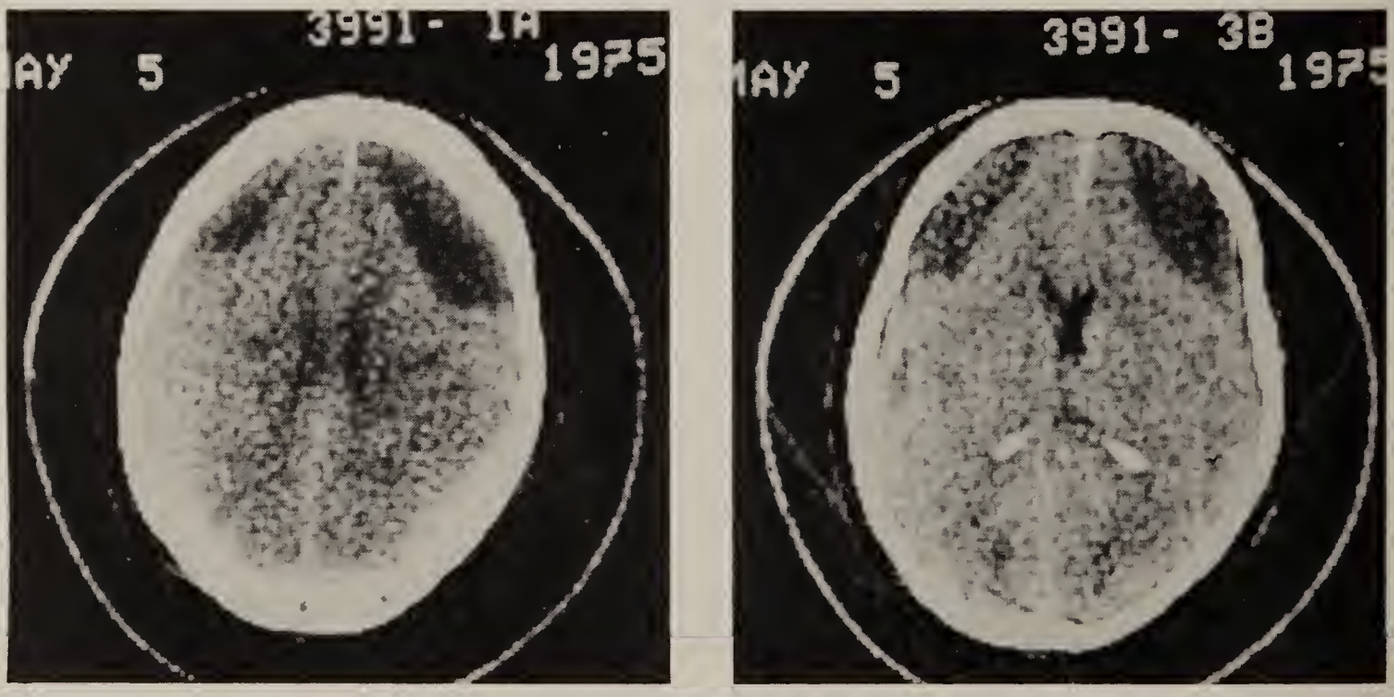


Fig. 11—Bilateral frontal chronic subdural hematomas.

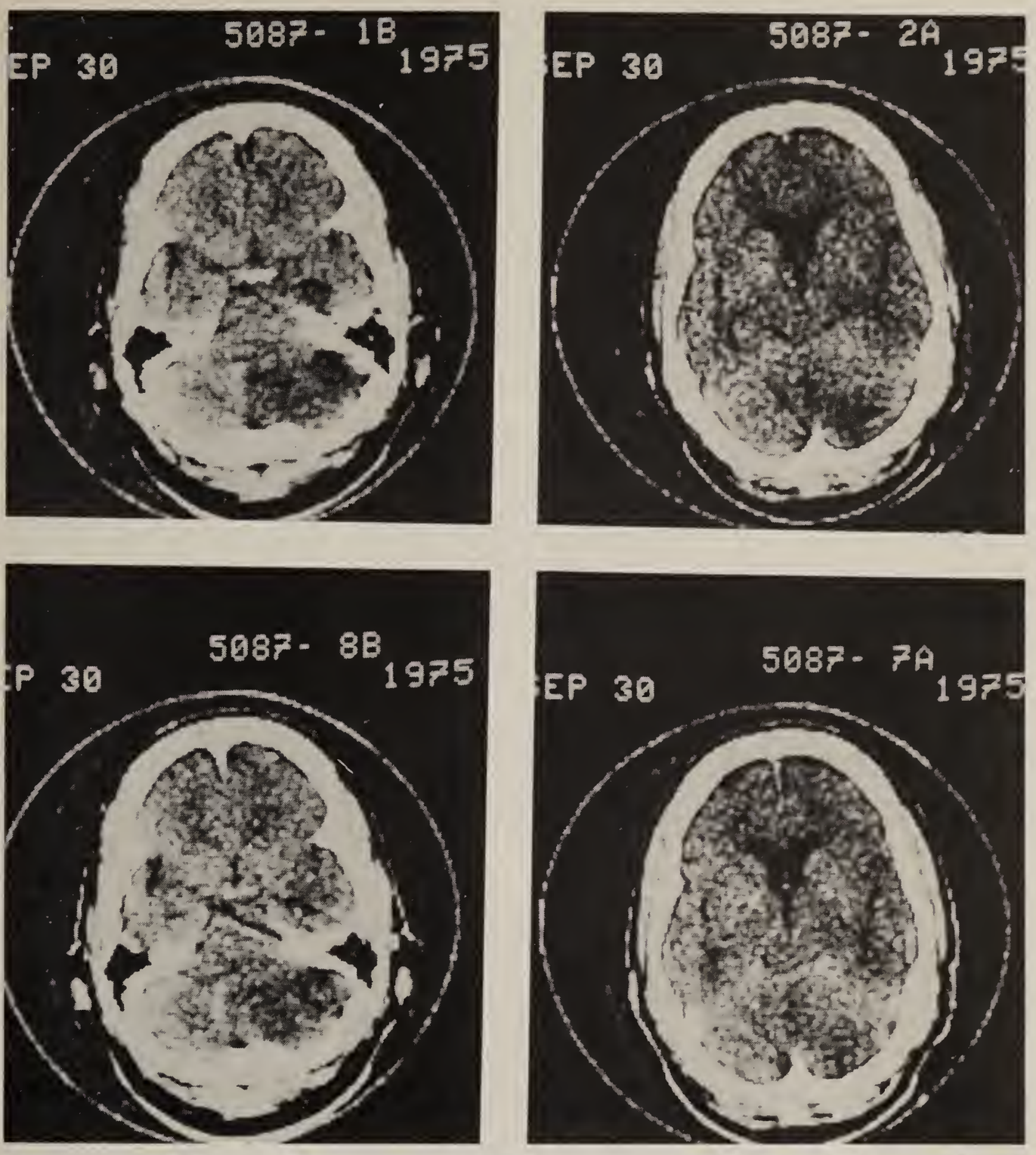


Fig. 12—Infarct right cerebellum. At angiography, both vertebral arteries were occluded. (1B, 2A: pre-infusion. 8B, 7A: post infusion.)

pheres) can be diagnosed when a large, single ventricle and small amount of neural tissue are present (Fig. 13). Marked generalized enlargement of the lateral and third ventricles with a normal-sized fourth ventricle, gives strong evidence for stenosis of the aqueduct of Sylvius in a teenage girl (Fig. 14). Localization of the level of obstruction will aid in the performance of pneumography which will still be needed to identify small lesions. Once a patient has had ventricular shunting, computed tomography is a precise and simple means of determining shunt function. Non-obstructive (communicating) hydrocephalus can be suggested when there is generalized ventricular enlargement and no evidence of cerebral atrophy. Further studies are necessary to make this diagnosis.

Atrophy

The low density of cerebrospinal fluid allows the size of the ventricles and sub-

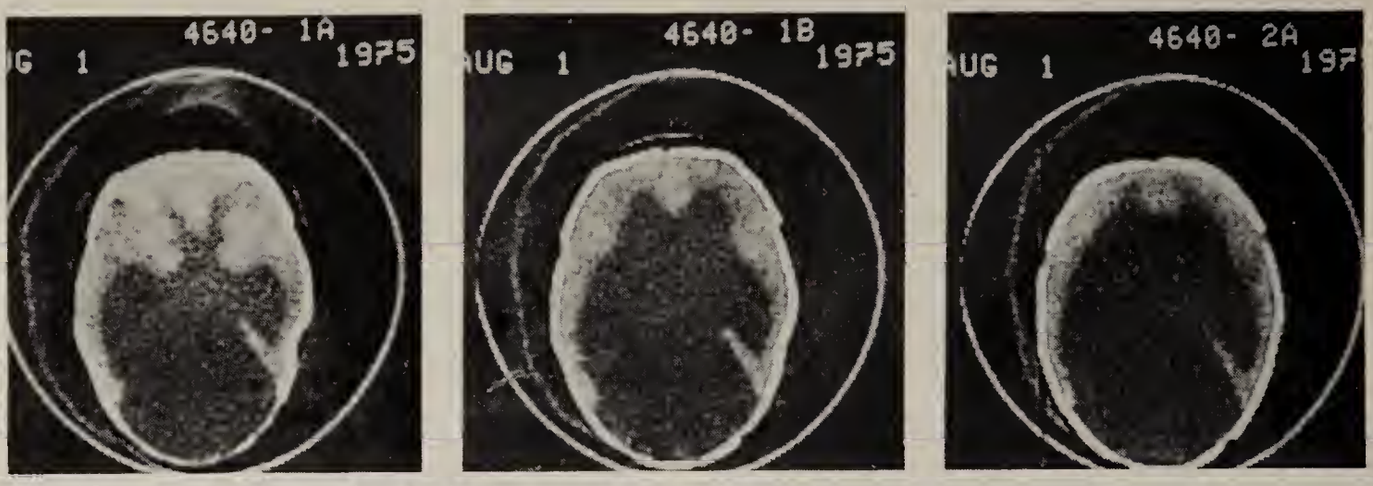


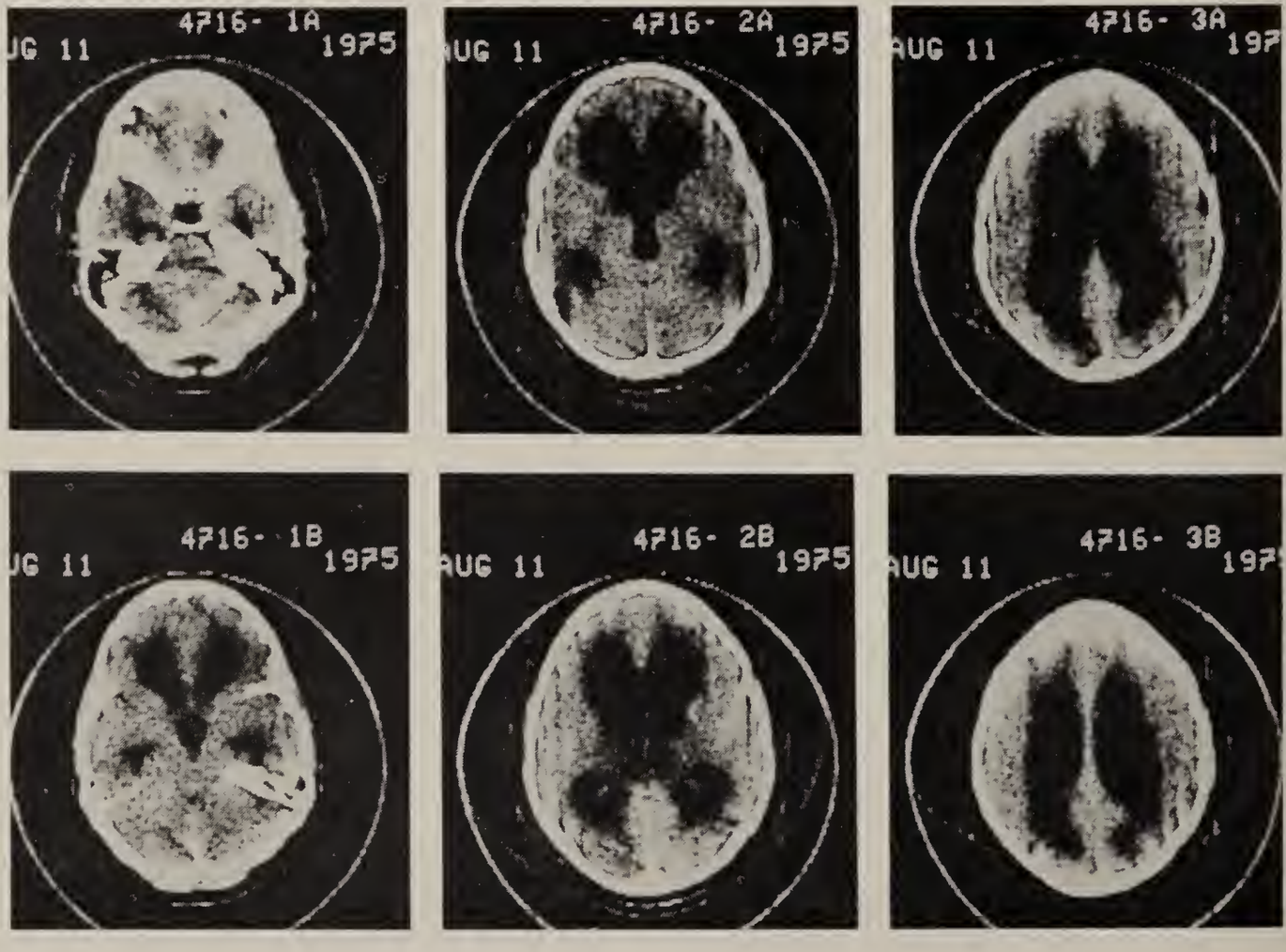
Fig. 13—Hydranencephaly. A single, large ventricle markedly compresses the under-developed brain.

arachnoid spaces to be easily determined. Localized areas of brain atrophy which may result from trauma, vascular insults or surgical intervention are readily discerned. Generalized brain atrophy, accompanied as it is, by enlargement of the ventricles, cortical sulci, sylvian fissures, and basal cisterns is readily and accurately determined (Fig. 15). Demented patients who do not have marked atrophy on computed tomography should be studied for potentially treatable causes of dementia.[6]

SUMMARY

Computed tomography of the head is a non-invasive screening technique which assists in the differentiation of a wide

Fig. 14—Aqueductal stenosis.



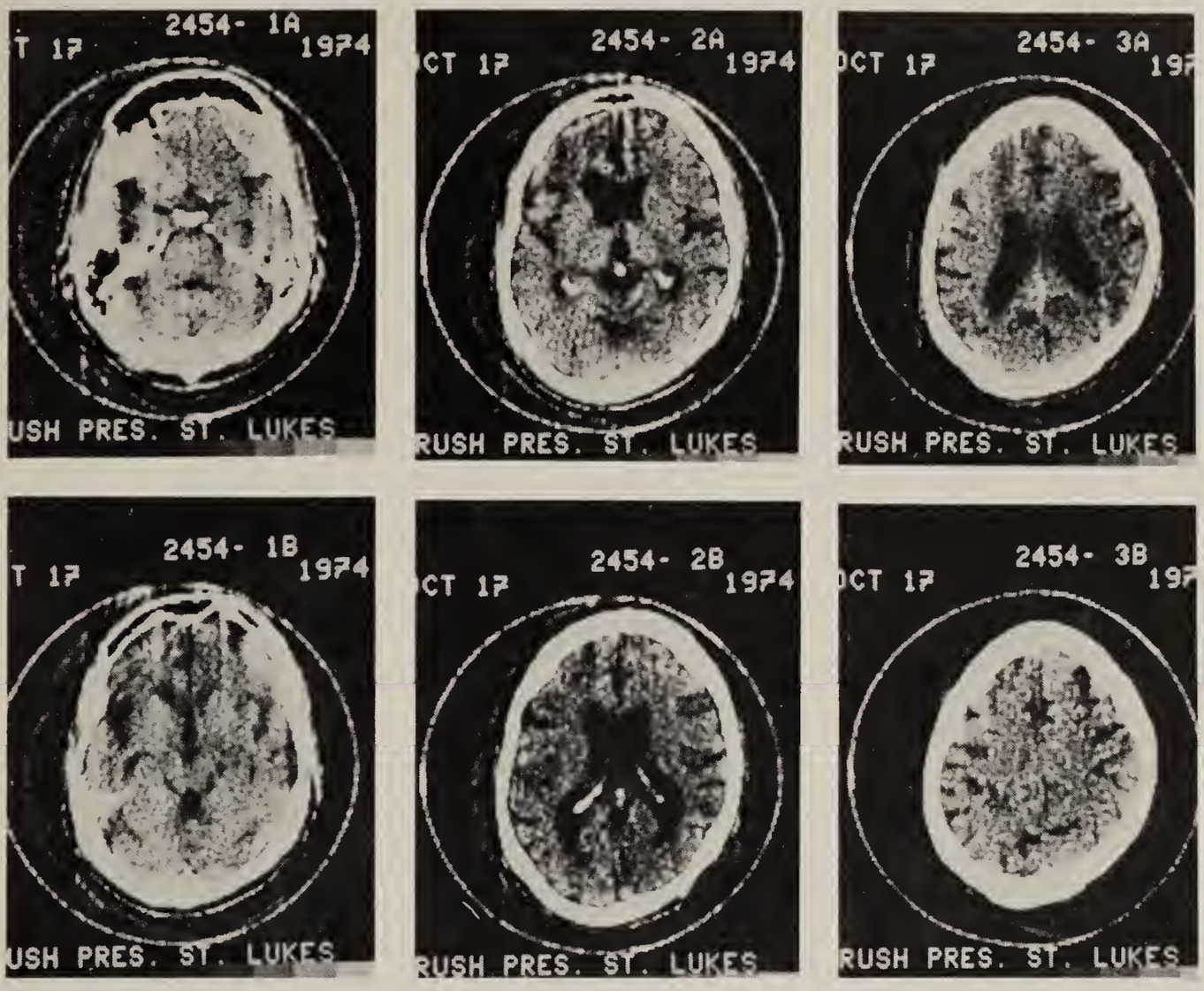


Fig. 15—Generalized cerebral atrophy.

spectrum of neurological diseases without discomfort to the patient. The only hazard is possible untoward reaction to iodinated contrast, the same risk as with a drip-infusion excretory urogram. In the near future, technical improvements will shorten the scanning time and thereby lessen artifacts caused by patient motion, and high-resolution matrices will produce better images.

REFERENCES

1. **Ambrose J:** Computerized transverse axial scanning (tomography) II. Clinical application. *Br J Radiol 46:1023, 1973*

2. **Houndsfield GN:** Computerized transverse axial scanning (tomography): I. Description of system. *Br J Radiol 46:1016, 1973*

3. **Huckman MS:** Clinical experience with the intravenous infusion of iodinated contrast material as an adjunct to computed tomography. *Surg Neurol 4:297, 1975*

4. **New PJF, Scott WR, Schnur JH, et al:** Computed tomography with the EMI scanner in the diagnosis of primary and metastatic intracranial neoplasms. *Radiology 114:75, 1975*

5. **New PJF, Scott WR, Schnur JA, et al:** Computerized axial tomography with the EMI scanner. *Radiology 110:119, 1974*

6. **Huckman MS, Fox J, Topel J:** The validity of criteria for the evaluation of cerebral atrophy by computed tomography. *Radiology 116:85, 1975*

FORCED OSCILLATIONS OF THE ANKLE JOINT

Gyan C. Agarwal
Gerald L. Gottlieb

ABSTRACT. Low-frequency oscillations in the frequency range of 3 to 30 Hz were applied to the ankle joint, superimposed upon a moderate tonic contraction of the triceps surae. **Among the phenomena observed are a very sharp increase in ankle compliance at about 6.5 Hz, the development of clonus at about the same frequency in fatigued but otherwise normal subjects, and a pronounced nonlinear rotational response of the foot to sinusoidal torques in the 8 to 12 Hz range. These phenomena, while presumably arising from fundamental stretch reflex mechanisms, cannot be explained without speculating on the nature of supraspinal regulation of spinal reflex mechanisms.**

INTRODUCTION

There has been a growing concern in recent years regarding the effects of vibration upon human operators. Nearly everyone is exposed at one time or another to some form of vibration, and it is conservatively estimated that there are eight million workers exposed to some form of industrial vibration in the United States alone.[1] Wasserman and Badger have compiled a bibliography of the literature on the effects of vibration.[2]

Two forms of vibration are of essential interest. The first is called whole body vibration and is the application of vibration, usually to the entire body applied from head to toe, as from a vibrating floor. The second is called segmental vibration and is the application of vibration to specific body parts, such as the hands, from vibrating hand tools.

Our knowledge of the direct effects of vibration on the human motor system is very limited. Long-term exposures in workers produce many pathological syndromes.[2,3] Vibration is a potent stimulus to the muscle spindles,[4-6] and therefore is potentially capable of producing significant changes in the control and coordination of movements.

Vibration in the frequency range of 50 to 200 Hz applied directly to the muscle belly or muscle tendon of the gastrocnemius-soleus muscle produces the tonic vibration reflex and significantly influences the tendon-jerk and Hoffmann reflexes.[7-11] Recent studies have shown kinaesthetic illusions arising from the stimulation of intramuscular receptors by vibration at 100 Hz with a physiotherapy vibrator.[12,13]

In this paper we will examine the effects of low-frequency (3 to 30 Hz) forced oscillation of the ankle joint. The moment of inertia of the limb presents an inertial load to an applied torque, the muscles offer a visco-elastic resistance to lengthening, and in addition to these, movement may be affected by active muscle contraction, both reflex and voluntary. In practice it is difficult to separate and

From the Department of Biomedical Engineering, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois, and the College of Engineering, University of Illinois at Chicago Circle, Chicago, Illinois

Gyan C. Agarwal, Ph.D., Visiting Professor, Department of Biomedical Engineering, Rush Medical College, Chicago, Illinois

Gerald L. Gottlieb, Ph.D., Associate Professor of Biomedical Engineering, Associate Professor of Physiology, Rush Medical College

Presented at the International Occupational Hand-Arm Vibration Conference, National Institute for Occupational Safety and Health, Cincinnati, Ohio, October 28-31, 1975

measure these three resisting forces. The inertia of the limb probably remains fairly constant. The resistance of muscles to lengthening and the intensity of the stretch reflexes are dependent on the force of contraction.

Joyce, Rack and Ross[14] have studied forced oscillations of the human elbow joint. Most of their results were similar to those reported here. However they did not study single frequencies, and the total frequency range up to 22 Hz was scanned in less than 30 seconds. Berthoz and Metral,[15] and Neilson[16] have used similar techniques of applying a sinusoidally varying force while measuring elbow position. Walsh[17,18] applied sinusoidally varying forces to the wrist and observed resonance and jump phenomenon. He scanned the total frequency range up to 15 Hz in 10 seconds. The results presented here show that these frequency scanning techniques fail to reveal a number of interesting behavioral phenomena.

METHODS

Experiments were done on six normal human subjects. The subject sat in a chair with his right foot strapped to a footplate which permitted only dorsiflexion-plantarflexion about the ankle joint. A schematic of the equipment used is shown in Fig. 1.

The plate is rotated by a D.C. torque motor via a gearbelt and pulley system for torque amplification. Constant tension springs (not shown in the figure) are also used to balance the downward gravitational force on the foot. With the motor off and the subject completely relaxed, the resulting joint position (approximately 90° between the foot and the tibia) is defined as the zero angular position, and this reference is provided as a fixed dot on a dual beam oscilloscope. The second beam is used to display the subject's true angular position.

The subject was instructed to try to maintain a constant mean force against the bias torque of the motor so that the oscillation was nearly symmetrical with respect to the reference position.

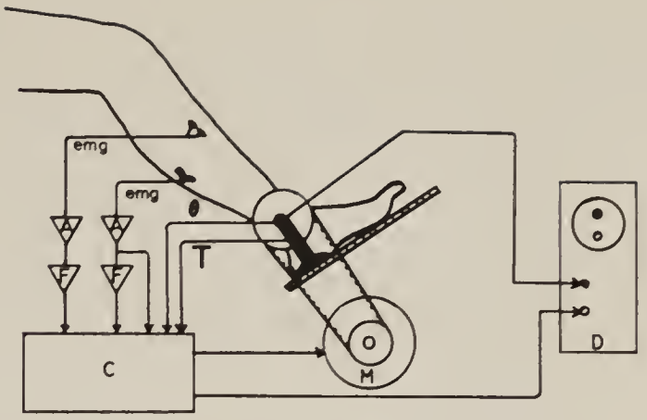


Fig. 1—A schematic of the appartus used for the forced oscillations of the ankle joint. The components are: D.C. Torque Motor (M) driven by a Bulova power amplifier, electromyogram is recorded using disc surface electrodes placed over the bellies of the soleus and anterior tibial muscles, EMG amplifiers (A) are Tektronic 2A61 (bandwidth 60-600 Hz), filters (F) are third order averaging (10 msec averaging time), display oscilloscope (D) is a dual beam Tektronix 502, digital computer (c) is General Automation SPC-16.

The torque was measured by four strain gauges on the side arms of the footplate and connected in a bridge circuit. The angular rotation was measured by a continuous potentiometer. Electromyograms were recorded from disc surface electrodes placed over the bellies of the soleus (GS) and anterior tibial (AT) muscles. These were amplified, full-wave rectified and filtered before recording.[19,20]

Sinusoidal signals were superimposed on the mean torque level. Frequencies from 3 to 30 Hz were used. In some experiments, frequencies down to 1 Hz were used. The torque, the resulting angular rotation and the electromyograms were continuously recorded on a digital tape. The angle and the torque signals were sampled at a rate of 250 Hz, and the filtered EMGs at a rate of 500 Hz. The data were continuously recorded for 10 seconds or more at each frequency. After 10 seconds, we frequently recorded the data going through a stop, start, and stop again of the modulating signal. The bias voltage was constant throughout the run. This allowed us to observe self-generated oscillations as discussed under Results.

Whereas the applied torque signal was nearly a single frequency sinusoid, the angular rotation at certain frequencies had

significant distortion. For this reason the following analyses were done:

1. Fourier coefficients at the fundamental frequency were obtained from the torque, the angular rotation and the EMG data for the first 10 seconds. The analysis was done for twenty half-second data records, and the resulting numbers were averaged.
2. A two-cycle time average was generated for a 10-second data record by taking successive intervals equal to twice the modulation period.
3. An average Fourier Transform was obtained by using five 2.048 seconds (512 points) data records with the incremental resolution frequency of 0.4883 Hz.

In some experiments, oscillation near the resonant frequency was applied continuously for 100 seconds or more to develop muscle fatigue and to observe the self-generated oscillations after the modulating signal of the motor was stopped.

RESULTS

The two-cycle averages which define the average wave for the torque, the angular rotation, and the two EMGs are shown in Fig. 2 at eight drive frequencies. The motor drive was $0.5 + 0.4 \sin \omega t$. volts which required tonic contraction of the gastrocnemius-soleus muscle to counteract the torque motor bias.

There are a number of interesting observations in these two-cycle averaged responses. As the frequency is changed from 3 to 30 Hz, the amplitude of the measured torque (differential torque between the motor and the foot torque) passes through a minimum near 6.5 Hz, and the amplitude of rotation is near maximum at these frequencies. At low frequencies, the anterior tibial muscle has no measurable EMG activity. (The constant value of AT EMG at 4 Hz was just the offset of the filtering amplifier). Near resonance, both soleus and AT are rhythmically active. At 12 Hz, the angular rotation is significantly distorted from the sinusoidal shape. Some distortion is also present in the torque wave-form. Alternating waves of the GS EMG are nearly suppressed.

Although Fig. 2 clearly points out the nonlinear nature of the system, as a first-order approximation, a linear analysis was performed. Fourier coefficients at the drive frequencies were computed, and their amplitudes are given in Table I.

The effective compliance of the muscle is defined by taking the ratios of the angular rotation and the torque Fourier coefficients. If R denotes the radius of action of the muscle, then

$$\frac{\Delta L}{\Delta F} \Delta = \text{Effective compliance of the muscle}$$

$$= \Theta \times R/(\tau/R)$$

$$= (\Theta/\tau)R^2 \text{ meters/newton}$$

where the angular rotation Θ is in radians and torque τ is in newton-meters. For plantar as well as for dorsal movements, the radius of action R is roughly 5 cm, although it varies slightly with the foot angle.[21]

The center of Fig. 2 shows the effective compliance as a function of the drive frequency. Also shown is the soleus EMG/stretch gain.

Fig. 3 shows the Fourier transforms of the same data. The distortion of the angular rotation at 10 and 12 Hz is manifested by subharmonic components. The center of Fig. 3 shows the phase angles between the angular rotation and torque and between the soleus EMG and the angular rotation. The sudden change in phase at 6.5 Hz in the Θ-τ curve is indicative of the resonance at that frequency. The EMG-Θ phase can be accounted for by a neural transport delay of about 55 msec.

Fig. 4 shows the slowly increasing amplitudes of oscillation when modulation is turned on near the resonant frequency of 6.5 Hz. The peak EMG of the gastrocnemius-soleus muscle also increases in amplitude as the oscillation builds up.

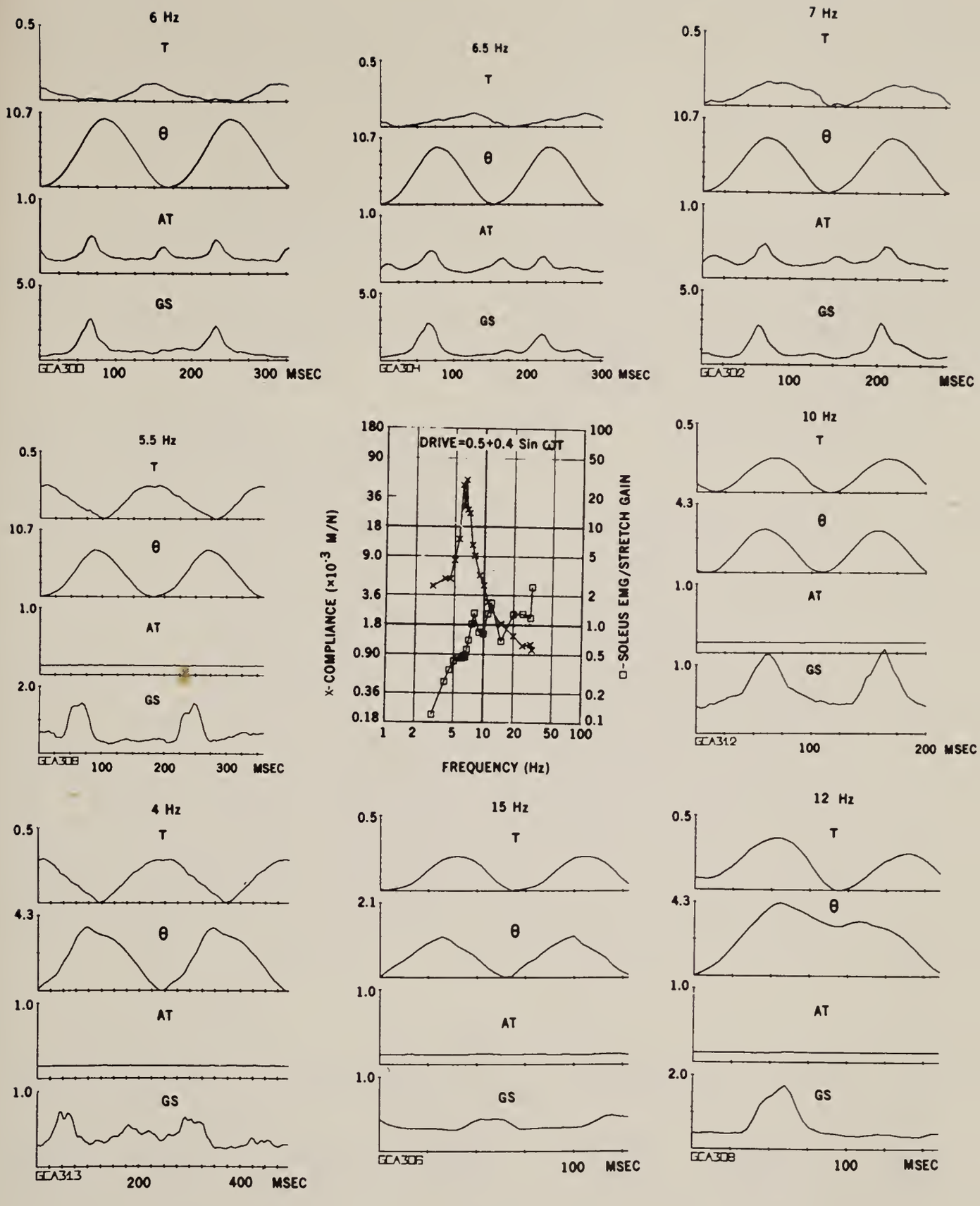


Fig. 2—Imposed sinusoidal movement of the ankle joint. Figure shows the two-cycle averaged response at eight drive frequencies. The four traces from top to bottom in each part are torque in kg-meters, foot angle in degrees, rectified and filtered EMG from the anterior tibial and the gastrocnemius-soleus muscles. The averaging was done for a 10 second data record by taking successive intervals equal to twice the modulation period. The motor drive was 0.5 + 0.4 Sin ωt. The center figure shows the effective compliance (x) in meters/newton and soleus EMG/stretch gain (□) in arbitrary units as a function of the input frequency. These were calculated from the Fourier analysis at the drive frequency. The effective lever arm of the muscles is assumed to be 5 cm.

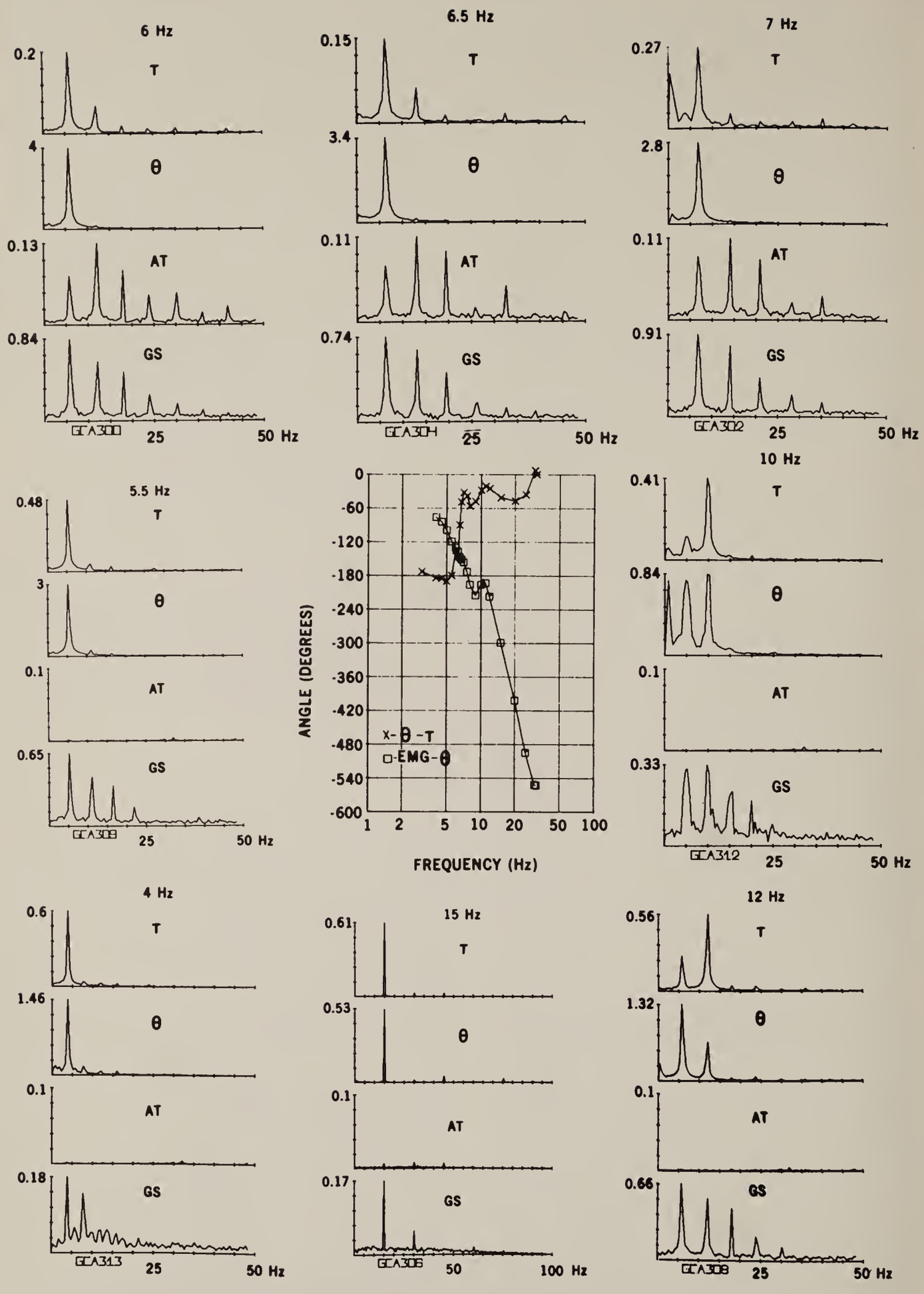


Fig. 3—Imposed sinusoidal movement of the ankle joint. Figure shows the Fourier transform of the torque (Kg-meters), angular rotation (degrees) and rectified, filtered electromyogram recorded from the anterior tibial and the gastrocnemius-soleus muscles at eight driving frequencies. The motor drive was 0.5 + 0.4 Sin ωt. The center figure shows the phase angle between angular rotation and the applied torque (x) and between the soleus EMG and the angular rotation (□) as a function of the drive frequency. The phase relationship was calculated from the Fourier analysis at the drive frequency.

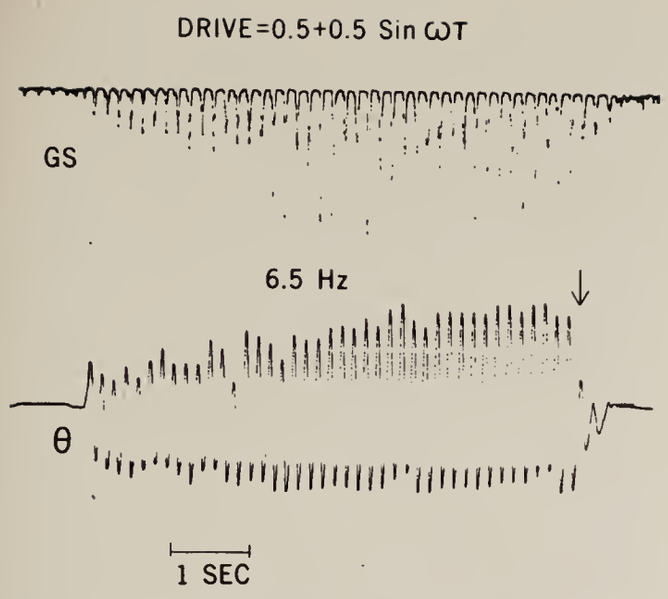


Fig. 4—Slowly increasing amplitude of oscillation near the resonant frequency of 6.5 Hz. The upper trace is the EMG of the gastrocnemius-soleus muscle (rectified and filtered) and the lower trace is the angular rotation. The drive was 0.5 + 0.5 Sin wt.

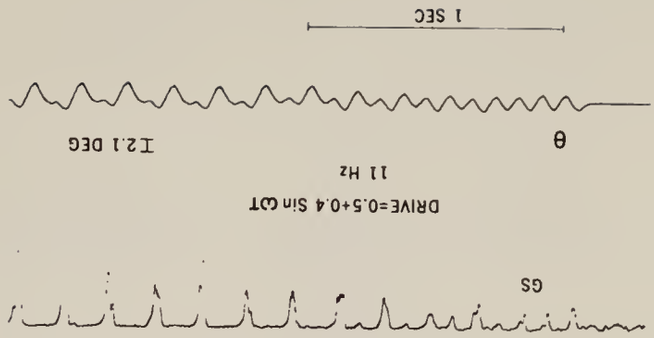


Fig. 5—Forced oscillation of the ankle joint at 11 Hz. The upper trace shows the EMG activity of the gastrocnemius-soleus muscle (rectified and filtered) and the lower trace shows the angular rotation. The time markers are one second apart. For the first one second, the angular rotation is at 11 Hz and then gradually the nonlinear terms become prominent.

Figs. 5 and 6 show the distortion in the angular rotation. In Fig. 5, the drive frequency is 11 Hz and the oscillation starts out at the driving frequency with corresponding EMG. Due to time-varying changes in the muscle's stiffness as the muscle contracts during each cycle, nonlinear behavior becomes progressively dominant with alternate stretch cycles becoming less prominent. The EMG pulses are also at half the driving frequency.

In Fig. 6, the driving frequency is 10 Hz. The spontaneous recurrences of oscillation at the driving frequency (indicated by underlines) with corresponding 10 Hz frequency in the soleus EMG, are observed for a few cycles between periods of nonlinear oscillation.

Fig. 6 also shows the autonomous oscillation of the foot after the modulating signal to the torque motor is turned off as indicated by the arrow. This free oscillation for the first two seconds after modulation is stopped is at 6.15 Hz as determined by Fourier transform analysis shown in Fig. 7. Such self-sustained oscillations are always near the resonant frequency and not at the drive frequency.

TABLE I

Fourier coefficients of the data in Figure 2. The motor drive was 0.5 + 0.4 sin $2\pi ft$. FC denotes the Fourier coefficient at the drive frequency and DC is the average value. (Numbers have been rounded to two digits)

FREQ Hz	GS-EMG FC	GS-EMG DC	Rotation (deg) FC	Torque (kg.M.) FC	Torque (Kg.M.) DC	Compliance (M/N) x 10^{-3}
4	0.19	0.39	1.6	0.12	0.16	5.3
5	0.51	0.51	2.6	0.13	0.19	8.2
5.5	0.76	0.63	3.5	0.11	0.19	13.5
6	1.0	0.78	4.7	0.04	0.2	47.5
6.25	0.9	0.74	4.0	0.06	0.18	28.7
6.5	0.95	0.77	4.4	0.03	0.18	54.3
6.75	0.95	0.76	3.7	0.06	0.2	26.7
7	1.2	0.95	3.8	0.06	0.19	24.8
8	1.0	0.80	1.7	0.08	0.53	9.0
10	0.5	0.61	1.3	0.12	0.17	4.6
12	0.63	0.66	0.81	0.13	0.18	2.5
15	0.17	0.37	0.53	0.12	0.20	1.8
25	0.19	0.34	0.33	0.13	0.17	1.1
30	0.16	0.34	0.3	0.11	0.16	1.1

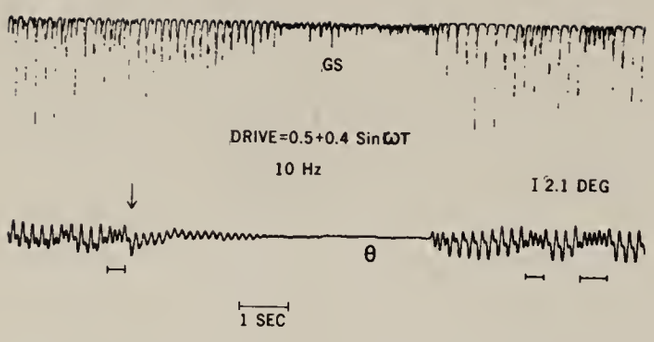


Fig. 6—Forced oscillation of the ankle joint at 10 Hz. The upper trace shows the EMG activity of the gastrocnemius-soleus muscle (rectified and filtered) and the lower trace shows the angular rotation. The time markers are one second apart. The arrow indicates the time when the modulation signal of the motor was turned off. The self-sustaining oscillation of the ankle joint continued for several seconds near 6.15 Hz. As the modulation signal was turned on again, the nonlinear wave form rapidly developed. The recurrence of 10 Hz oscillations between the nonlinear response are indicated by line segments underneath the angular rotation curve.

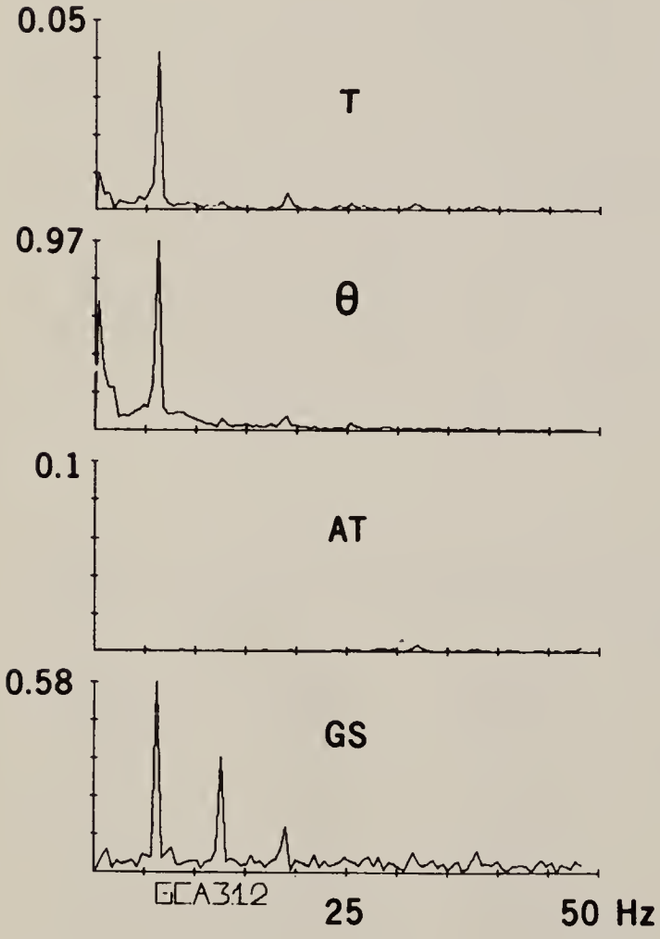


Fig. 7—Fourier transform of the data for 2 seconds after the modulating motor signal was turned off as shown in Figure 6. The four traces from top to bottom are torque (Kg-meters), angular rotation (degrees), EMG of anterior tibial and gastrocnemius-muscles. The frequency of the self-sustaining oscillation was 6.15 Hz.

(The nearest drive frequencies tested were 5.5, 6, 6.25 and 6.5 Hz.) These oscillations are *not* voluntary in nature.

Figs. 8 and 9 show the muscle compliance for six experiments when the amplitude of modulation was kept constant and the bias voltage changed from —0.5 to 0.75 volts. For the relaxed foot and zero motor bias voltage, the resonant frequency is at 4 Hz. For tonically active muscles, the resonant frequency is around 5.5 to 6.25 Hz.

Fig. 10 shows the muscle compliance for the case when the bias is kept constant at 0.5 volts and the amplitude of modulation is varied with values of 0.2, 0.4, 0.5 and 0.6 volts. At 0.2 volts modulation, two peaks in the compliance curve are seen at 9 and 12 Hz; at 0.4 volts modulation two peaks are seen at 6 and 6.5 Hz; at 0.5 volts, one peak is seen at 6.25

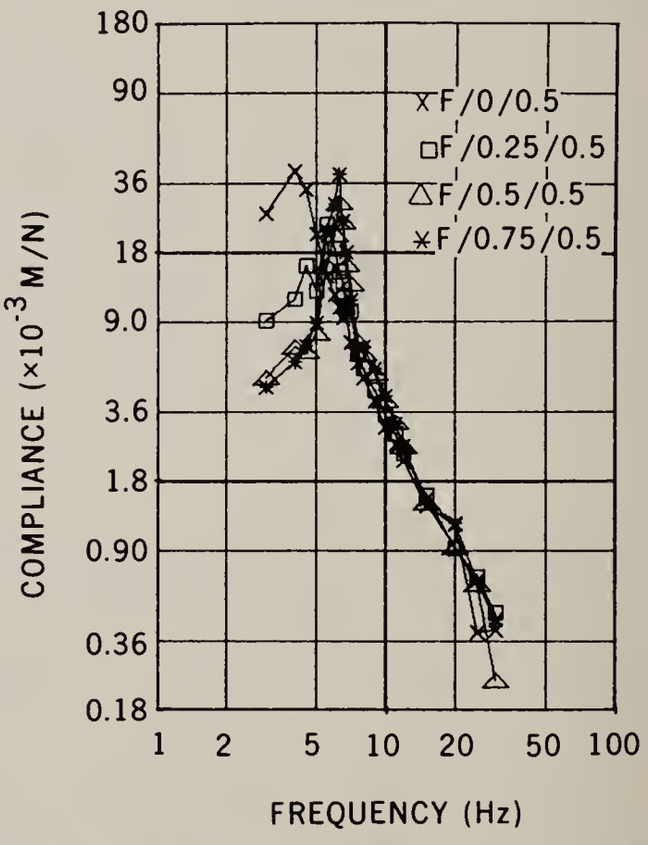


Fig. 8—Effective compliance (angular rotation/torque Fourier Coefficients) in meters/newton as a function of the input frequency. The amplitude of the modulation signal to the motor was kept constant at 0.5 volts. The motor bias voltages for the four cases were: x 0 volts, □ 0.25 volts, △ 0.5 volts, and * 0.75 volts. For positive non-zero motor bias the gastrocnemius-soleus muscle was tonically active to maintain the zero angular foot position. The resonant frequencies for these four cases were 4, 5.5, 6.25, and 6.75 Hz, respectively. (Subject GLG).

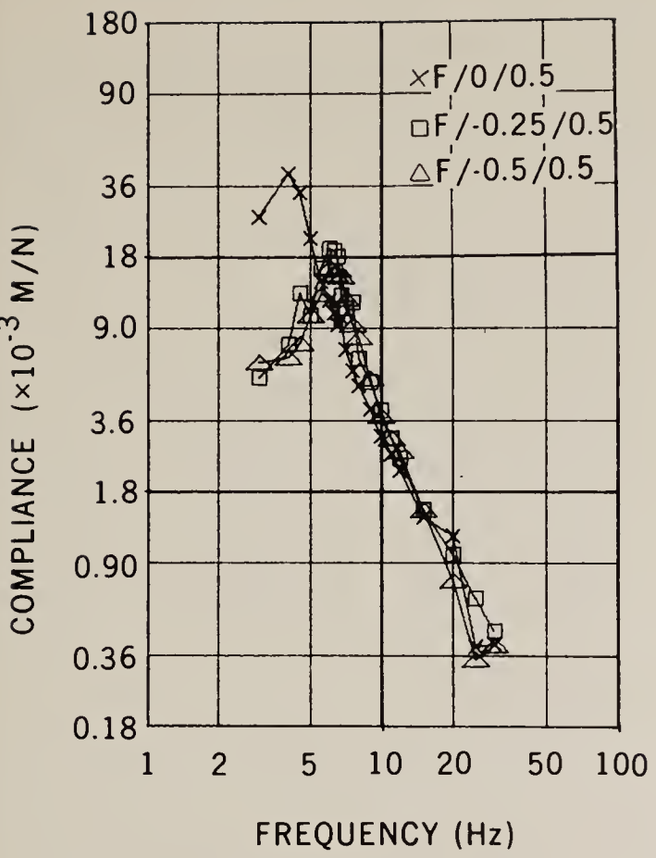


Fig. 9—Effective compliance (angular rotation/torque Fourier Coefficients) in meters/newton as a function of the input frequency. The amplitude of the modulation signal to the motor was kept constant at 0.5 volts. The motor bias voltage for the three cases were: x 0 volts, □ −0.25 volts, and △ −0.5 volts. For negative non-zero bias the anterior tibial muscle was tonically active to maintain the zero angular foot position. The resonant frequencies for these three cases were 4, 6, and 6 Hz, respectively. (Subject GLG).

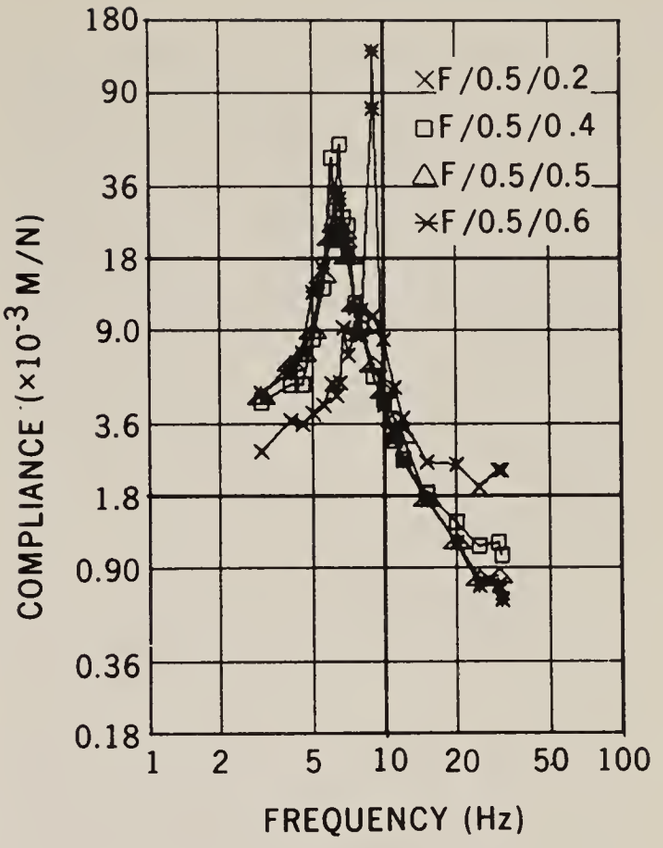


Fig. 10—Effective compliance (angular rotation/torque Fourier Coefficients) in meters/newton as a function of the drive frequency. The motor bias voltage was kept constant at 0.5 volts. The amplitude of the modulation signal for the four cases was: x 0.2 volts, □ 0.4 volts, △ 0.5 volts, and * 0.6 volts. The gastrocnemius-soleus muscle was tonically active against the motor bias to maintain the zero angular foot position. The resonant frequencies for these cases were: 6.75 and 8 Hz at 0.2 volts modulation, 6 and 6.5 Hz at 0.4 volts modulation, 6.25 Hz at 0.5 volts modulation, and 6.25 Hz and 9 Hz at 0.6 volts modulation. (Subject GCA).

Hz and at 0.6 modulation the two peaks are seen at 6.25 and 9 Hz.

Fig. 11 shows the angular and torque power ratios defined as the ratio of power near the drive frequency divided by the total power of the signal. This ratio was obtained from the Fourier transform data by taking the sum of square of five frequency points nearest the drive frequency (± 1 Hz band) divided by the total sum of the squares which represents the total power. In the range of 8 to 12 Hz, significant power of the angular rotation is in the subharmonics.

DISCUSSION

Figs. 2, 3, 5 and 6 show the nonlinear nature of the system over the frequency range of 8 to 12 Hz. In earlier studies[14,17,18] these nonlinear effects were

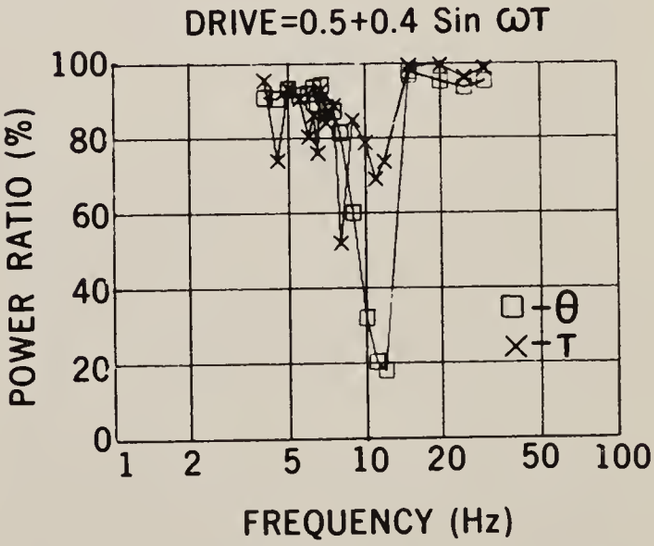


Fig. 11—Angular rotation and torque power near the drive frequency as a function of the total signal power.

not observed because of their frequency sweeping technique. As illustrated in Fig. 5, the nonlinear oscillation does not develop instantly. The details of this behavior have not yet been fully investigated. The generation of subharmonics at these frequencies is most likely due to time-varying changes in the compliance of the rhythmically contracting muscle. The compliance of the human arm varies with contraction and as calculated from Wilkie's data is from 0.5×10^{-3} to 1.5×10^{-3} meters/newton.[22,23]

Normal physiological tremor has a frequency range of 8 to 12 Hz Lippold[24] concluded that physiological tremor is due to oscillation in the stretch reflex servo-loop. One cannot exclude the possibility that the mechanisms responsible for physiological tremor interact with forced oscillatory inputs in the frequency range to produce these nonlinear modes of behavior.

The behavior of the system in Fig. 6 is most interesting. The recurrences of 10 Hz oscillations between the nonlinear response suggests that the system was near the boundary of linear and nonlinear regions. This behavior, coupled with the existence of self-sustaining oscillations which are emphasized with fatigue (in one experiment a subject continued to oscillate for 58 seconds until he finally halted it) is surprising. The self-sustaining oscillations have been seen by Joyce et al.[14] in the elbow movement. Such oscillations are due to the regenerative effects of the feedback loop and imply instability of the loop.

Conservative engineering design tends to emphasize stability, and this normally characterizes our view of most physiological regulating mechanisms. An alternative view of many such regulators is that they are inherently unstable within some of their inner loops. Homeostasis is preserved however by the existence of outer loops which become active only near the boundaries of some allowable state space. Thus the inherent instability is not observed except in cases of pathology or perhaps in experiments such as described here. Certainly none of our subjects has any history of neuromuscular illness nor has any present complaints. None shows ankle tremor and none has difficulty walking or driving, but all have experimentally demonstrated clonus. This is a most interesting paradox.

The slow buildup in the amplitude of oscillation near the resonant frequency (see Fig. 4) is a common phenomenon in nonlinear systems. The "jump" phenomenon reported by Walsh[18] near the resonant frequency of the wrist movement is commonly seen in mechanical systems with mass, damper and a stiff spring. Such a system is described by Duffing's equation.[25]

In Figs. 8 and 9, the resonance of the relaxed foot is near 4 Hz. With tonic activity the resonance is seen near 6 to 6.5 Hz. The resonant frequency is also dependent on the amplitude of modulation as seen in Fig. 10. At 0.2 modulation, the resonance is seen near 8 Hz. At 0.4 and 0.5 modulation, the resonance is near 6.25 to 6.5 Hz. At higher modulation of 0.6, resonance is seen at 6.25 and 9 Hz. The second resonance which is in the range of physiological tremor could likely be due to the stretch reflex mechanism.

These observations show the complexity of the peripheral stretch reflex system. Use of forced oscillations as the input to study these mechanisms provides an interesting avenue of investigation. Such investigations are of particular relevance to man-machine systems and job safety.

ACKNOWLEDGEMENT

This work was supported in part by a National Science Foundation grant #GK-37540 and by General Research Support Grant #RR-05477 from the National Institutes of Health.

REFERENCES

1. **Wasserman DE, Badger DW, Doyle TE, Margolies L:** Industrial vibration — an overview. *Amer Soc Safety Eng J 19:38, 1974*

2. **Wasserman DE, Badger DW:** Vibration and the Worker's Health and Safety. *USDHEW Nat Inst Occ Safety and Health, Report 77, 1973*

3. **Taylor W:** Ed., *The Vibration Syndrome.* London, Academic Press, 1974.

4. **Bianconi R, Van der Meulen JP:** The response to vibration of the end-organs of mammalian muscle spindles. *J Neurophysiol 192: 177, 1963*

5. **Brown MC, Engberg I, Matthews PBC:** The relative sensitivity to vibration of muscle receptors of the cat. *J Physiol 192:773, 1967*

6. **Matthews PBC:** *Mammalian Muscle Receptors and their Central Actions.* Baltimore, Williams & Wilkins, 1972

7. **Hagbarth KE, Eklund G:** Motor effects of vibratory muscle stimuli in man. In *Nobel Symposium I—Muscular Afferents and Motor Control,* Ed. Granit R, Stockholm, Almquist & Wiksell, 1966, pp. 177-186

8. **De Gail P, Lance JW, Neilson PD:** Differential effects on tonic and phasic reflex mechanisms produced by vibration of muscles in man. *J Neurol Neurosurg Psychiat 29:1, 1966*

9. **Hagbarth KE:** The effect of muscle vibration in normal man and in patients with motor disorders. In *New Developments in Electromyography and Clinical Neurophysiology,* Ed. Desmedt JE, Vol 3, Basel: Karger, 1973, pp. 428-443

10. **Lance JW, Burke D, Andrews CJ:** The reflex effects of muscle vibration. In *New Developments in Electromyography and Clinical Neurophysiology,* Ed. Desmedt JE, Vol 3, Basel: Karger, 1973, pp. 444-462

11. **Agarwal GC, Gottlieb GL:** Effects of vibration on human spinal reflexes. In *Motor System-Neurophysiology and Muscle Mechanism,* Ed. Shahani M, Amsterdam: Elsevier, 1975 (in press)

12. **Goodwin GM, McCloskey DI, Matthews PBC:** The contribution of muscle afferents to kinesthesia shown by vibration-induced illusions of movement and by the effects of paralysing joint afferents. *Brain 95:705, 1972*

13. **McCloskey DI:** Differences between the senses of movement and position shown by the effects of loading and vibration of muscles in man. *Brain Res 63:119, 1973*

14. **Joyce GC, Rack PMH, Ross HF:** The forces generated at the human elbow joint in response to imposed sinusoidal movements of the forearm. *J Physiol 240:351, 1974*

15. **Berthoz A, Metral S:** Behavior of a muscular group subjected to a sinusoidal and trapezoidal variation of force. *J Appl Physiol 29:378, 1970*

16. **Neilson PD:** Frequency response characteristics of the tonic stretch reflex of biceps brachii muscle in intact man. *Med Electron Biol Eng 10.460, 1972*

17. **Walsh EG:** Motion at the wrist induced by rhythmic forces. *J Physiol 230:44P-45P, 1973*

18. **Walsh EG:** Resonance at the wrist—a "jump" effect. *J Physiol 245:69P-70P, 1974*

19. **Gottlieb GL, Agarwal GC:** Filtering of electromyographic signals. *Am J Phys Med 49: 142, 1970*

20. **Gottlieb GL, Agarwal GC, Stark L:** Interactions between the voluntary and postural mechanisms of the human motor system. *J Neurophysiol 33:365, 1970*

21. **Hogins MT:** Identification of the human ankle control system. Ph.D. Thesis, University of Illinois at Urbana, 1969

22. **Wilkie DR:** The relation between force and velocity in human muscle. *J Physiol 110: 249, 1950*

23. **Gottlieb GL, Agarwal GC, Stark L:** Stretch receptor models: single-efferent single-afferent innervation. *IEEE Trans. Man-Machine Systems 10:17, 1969*

24. **Lippold OCJ:** Oscillation in the stretch reflex arc and the origin of the rhythmical, 8-12 c/s component of physiological tremor. *J Physiol 206:359, 1970*

25. **Cunningham WJ:** *Introduction to Nonlinear Analysis,* New York, McGraw-Hill, 1958

LEADERSHIP IN NURSING IN 1975

LUTHER CHRISTMAN

Leadership is a quality that is hard to define, difficult to teach, and almost impossible to measure. Persons in leadership roles are frequently at risk because they often must advocate the unpopular, deal with entrenched interests, and constantly cope with ambiguity. In making the above statements, I am referring to "breakthrough" leadership as compared with the leadership of the elite and the *status quo* establishment of the various professions. The *status-quo* leaders have a relatively easy and safe role—they merely have to advocate more of the same in a bigger way.

Leadership success, in the final analysis, is the total value of the positive outcomes for the profession. These outcomes are the result of actions taken, or not taken, to bring about changes within the profession. The following suggested changes can be accomplished with nominal effort but probably will call for a substantial shift of attitude on the part of many members of the profession.

(1) The first is to develop nursing as an applied science. This endeavor will require nurses to have a much greater knowledge of the fundamental sciences, both biological and behavioral. Nurses will have to learn the cognitive style needed to transform the theory and content of science into a constructive empirical means of aiding clients and patients. The present "reflex" practice that is highly prevalent throughout the nation will have to be replaced by a more precise form of care. By reflex practice I mean taking care of patients by morning, afternoon, and evening routines and by policies, rules and procedures. This type of care has led to impersonal, non-patient-oriented care designs.

(2). A companion issue in the expansion of this enterprise is the development of perfect accountability. The method of accomplishing this objective is most promising in the move to primary nursing. When primary nursing is properly designed, the elements of perfect accountability and self-supervision are in place. Team nursing, which not infrequently is no more than large groups of nurses taking care of mobs of patients, will have to be abandoned. The stratification of workers around patients will be considerably reduced, decision-making decentralized, and errors unerringly fixed, but so also will the nurses doing quality care be recognized for their competence. Team nursing has disrupted continuity of care by the constant rotation of assignments. Furthermore, it has been a major cause of the disruption of communication with members of the other health professions because they are not able to follow the very frequent shifting of assignments. One outcome of this constant change of assignments has been erratic and incomplete feedback to others, as well as uncertainty about patients amongst registered nurses themselves.

(3). A third topic for consideration is that of developing an all-registered nurse staff. This undertaking is more accomplishable than many might think. It can be accomplished by shifting the emphasis of activities for nurses. Several years ago, Richard Jelinek and I published an article based on our conclusions about the data coming from an analysis of nurse activi-

Luther Christman, Ph.D., R.N., Vice-President for Nursing Affairs, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois; Dean, Rush College of Nursing, Rush University, Chicago, Illinois

Presented at the Rhode Island State Nurses' Association Convention, October 22, 1975, at Cranston, Rhode Island

ties. These data showed that nurses spent about 75 percent of their time in non-clinical activities. Taken to its ultimate conclusion, the data would indicate that we are inefficiently using 75 percent of registered nurse time. The reorganization of nursing time into a 100 percent clinical effort would supply all the nurse manpower necessary to achieve this goal. The presence of an all-registered nurse staff would permit the formation of much higher standards of nursing care, particularly if nurse specialists were on each unit as pacemakers for clinical practice.

(4). Measuring the abilities of nurses and developing a reward system based on levels of competency are more easily done with an all-registered nurse staff, but are not necessarily limited to this restriction. To provide incentives for nurses to continually upgrade their clinical skills, adequate economic rewards are a basic ingredient. We already have a model for this in the way that university faculty are compensated. The use of a levels-of-practice method of rewarding nurses means that the clumping together of nurses in the same payroll categories, regardless of the quality of their input, will no longer exist.

(5). It is becoming increasingly evident that nursing education and nursing service must be re-wed. The divorce of these two inseparable components of the profession has been an unhappy affair and the sooner we get them back together, the better will be the outcome for patients. The study done by a Rush-Medicus group of investigators showed that in the nineteen hospitals studied, there was not any correlation with the quality of nursing care by having a basic educational program utilizing the clinical facilities. It probably would be much easier to merge both education and practice if nursing education took place only in complete schools. By complete schools, I mean the presence of undergraduate education through to the doctoral level, continuing education to assist nurses to combat obsolescence, and clinical research to improve the quality of education. If this concept were adopted, we probably could phase out 90 percent of our schools and produce more and better nurses.

(6). In order to have a greater influence on the care process, nurses must seek new organizational patterns. One suggested way to attain this end is to organize professional nursing in a pattern similar to that of the physician attending staff. To do this means that nurses will have to organize a committee structure and elect a president of the staff to represent them to management and to the Board. Nurses will have to set standards for admission to staff, police practice, undergo review, and set the limits of practice for each nurse according to his/her preparation and demonstrated competence. Rigorous self-regulation will eventually gain credibility and influence. Professional behavior of this nature is a viable alternative to economic security programs.

(7). Finally, in this list of issues suggested as future-oriented leadership, I wish to suggest the development of centers of excellence in nursing as a major means of creating an empirical demonstration of what nurses are capable of doing with all the new knowledge available to them. Centers of excellence are defined as medical centers where there is a very high quality of clinical nursing care, where educational programs flourish in an outstanding way, where clinical research is being done to expand the horizons of nursing practice, and where continuing education and outreach demonstration projects are influencing the practice of nurses in the region.

In this brief list of possible agenda items for the next several years, I have not attempted to be exhaustive. Many persons in this audience may have more interesting and professionally stimulating concepts to suggest. All of us are intensely interested in helping the profession move forward. All professions have to be future-oriented in order to remain viable and to carry out the mandate given to them by society. To do less is to shortchange ourselves; but more importantly, it is to fail patients.

ABSTRACTS

OF PUBLICATIONS BY THE STAFF

Acupuncture

Sadove MS, Okazaki K, Kim SI, Lee MH, Liu TH: *Deafness and acupuncture. Ill Med J 3:105, 1974*

At present, after almost two years of limited activity in acupuncture therapy for deafness by the classical technique, we can make no statement but that we have failed in our initial activity to significantly improve patients. However, we are not satisfied and we cannot stop. We must study the new and more intensive techniques in all fairness to reach a correct conclusion. We sincerely believe that this must be studied in an adequate number of patients, preferably in a number of our state institutions or by a state or philanthropically sponsored study group.

Anatomy

Jasch LG, Schmidt AJ: *Isocitrate lyase activity in the regenerating forelimb of the adult newt. J Exp Zool 190:199, 1974*

Regenerating and non-regenerating limb tissues from the adult newt, *Diemichtylus viridescens,* were assayed for isocitrate lyase activity. The enzyme assays were performed by micromodifications of existing procedures. In general, the whole homogenate, or a soluble fraction of the homogenate, was incubated with the substrate isocitrate. Isocitrate is cleaved by isocitrate lyase to glyoxylate and succinate. At the termination of the reaction, the 2,4-dinitrophenylhydrazone derivative of glyoxylate was produced, extracted and quantitated spectrophotometrically. Isocitrate lyase activity was localized to bulb and two-digit regenerates. The reaction product, glyoxylate (a monocarboxylic keto acid), was of special interest due to its role as a potent *in vitro* metabolic inhibitor. Therefore, the endogenous level of monocarboxylic keto acids, and the ability of regenerating tissue to produce and accumulate monocarboxylic keto acids, were investigated. Whole homogenates of regenerating tissue always contained more monocraboxylic keto acids than intact forearm or stump tissue. Also, after 30 minutes incubation with buffer alone, only regenerating tissue, produced and accumulated additional monocarboxylic keto acids. In regenerating tissue, the isocitrate lyase reaction may be utilized in the metabolism of lipid. Since regenerating tissue can produce and accumulate monocarboxylic keto acids *in vitro,* glyoxylate produced by the isocitrate lyase reaction may accumulate *in vivo* and participate in metabolic regulation.

Johnson MC, Schmidt AJ: *Collagen synthesis in the regenerating forelimb of the adult newt,* Diemichtylus viridescens. *J Exp Zool 190:185, 1974*

The regenerating and stump tissues of amputated forelimbs of the adult newt, *Diemichtylus viridescens,* were analyzed for collagen content and synthesis. Collagen soluble and

insoluble in 0.45 M NaCl was assayed by (1) spectrophotometric determination of hydroxyproline, (2) radiotracer determination of ^{14}C-proline incorporation, and (3) radiochromatographic analysis of the ^{14}C-proline/^{14}C-hydroxyproline ratio in the two collagen fractions. The results obtained reveal that there is significantly less saline insoluble as well as soluble collagen in the regenerating limb tissues than in the basal stump of the amputee, though a soluble fraction is demonstrable throughout the course of regeneration. By ninety days after amputation, there is a significant increase in the soluble collagen fraction in the regenerating limb. The soluble collagen fractions assayed in the regenerating tissues are newly synthesized protein, and this synthetic activity appears to be greatest during the paddle and early digitiform stages of regeneration. The soluble collagen extracted from regenerates appears to be less completely hydroxylated with respect to hydroxyproline content than obtains in corresponding fractions from limb stump and control limb tissues.

Biochemistry

Bezkorovainy A: *Medical care in Tsarist Russia. Texas Rep Biol Med 32:621, 1974*

Pre-World War I Russia was faced with many problems in the realm of medical care, which were quite similar to those encountered in the underdeveloped areas of the world: its child mortality rates were very high, there was a prevalence of infectious disease, and there was a general shortage of physicians. The medical care system that was slowly emerging in Russia at that time was based on the co-existence of privately and publicly financed medical practice. The public medical care system was based on the concept of small medical districts, where each had a small dispensary and an out-patient clinic. Several such districts supported a large general hospital and a mental institution. Such districts had their own local taxing base with some support from the central government. The publicly financed system was, however, badly administered and received inadequate support, so that its full potential had not been realized at the time of the fall of the Russian Empire.

Kornel L, Miyabo S, Saito Z, Cha RW, Wu FT: *Corticosteroids in human blood. viii. Cortisol metabolites in plasma of normotensive subjects and patients with essential hypertension. J Clin Endocrinol Metab 40:949, 1975*

Results of our previous studies revealed a derangement in the peripheral metabolism of adrenal steroids in patients with essential hypertension. To investigate further this finding, all individual free and conjugated metabolites of cortisol were isolated, identified and quantitated in plasma of 14 normotensive subjects and 13 patients with benign, uncomplicated essential hypertension, following intravenous administration of a tracer dose of [4-^{14}C] cortisol. In addition, plasma levels of endogenous cortisol were determined at 8 a.m. and 4 p.m. in all the subjects examined. The results obtained revealed the following statistically significant differences between normotensives and hypertensives: 1) Mean plasma concentrations of cortisol metabolites reduced in ring-A with noreduced 20-ketone, tetrahydrocortisol, tetrahydrocortisone, and their 5α-epimers, were 30 percent *lower* in the hypertensives; since these steroids constitute the bulk of the major group of cortisol metabolites the glucuronide conjugates, plasma levels of this group of conjugates measured *in toto* were also found to be significantly *lower* in the hypertensives. 2) Concentrations of cortisol metabolites with nonreduced ring-A (Δ^4-3-keto configuration preserved) but with reduced 20-ketone and/or hydroxylated at C-6, 20α and 20β dihydrocortisol, 6α and 6β hydroxycortisol, and 6-hydroxy-20-dihydrocortisol (all 4

isomers), were 73 percent, 48 percent, and 68 percent respectively, *higher* in the hypertensives; since these steroids constitute the bulk of the sulfate-conjugated and nucleoside-complexed metabolites of cortisol, plasma levels of these group of metabolites, measured *in toto*, were also found to be *higher* in the hypertensives. No significant difference was found between normotensives and hypertensives in the a.m. and p.m. plasma levels of cortisol. These findings, in conjunction with the results of our studies on urinary corticosteroid metabolites, which yielded identical findings, provide evidence for a decreased activity of hepatic cortisol-$\triangle^4$-hydrogenase enzyme system and increased activities (presumably compensatorily) of cortisol-20-reductase and 6-hydroxylase enzyme systems in patients with essential hypertension. The interrelation of these findings with those of other investigators studying steroid metabolism in hypertension, points to the possibility that the detected aberration of activities of corticosteroid metabolizing enzymes may be an etiological factor in essential hypertension.

Zschocke RH, Bezkorovainy A: *Structure and function of transferrins. II: Transferrin and iron metabolism. Arzneim Forsch 24:726, 1974*

The first part of this review dealt with the physical, chemical, and iron-binding properties of transferrins, the nonheme iron-binding proteins of vertebrate plasma, mammalian milk, and avian egg-white. In the present part the biological functions of these proteins are discussed, placing special emphasis on the function of plasma transferrin in iron metabolism.

Plasma transferrin is the essential intermediate in iron metabolism, facilitating the utilization/re-utilization of iron for hemoglobin synthesis. Only iron bound to transferrin is taken up by the heme synthesizing erythroblasts. The exact mechanism of the iron transfer process remains largely unknown.

Cardiology

Levin AR, Liebson PR, Ehlers KH, Diamant B: *Assessment of left ventricular function in secundum atrial septal defect: evaluation by determination of volume, pressure, and external systolic time indices. Pediatric Research 9:894, 1975*

Left ventricular function and volume data from 17 control subjects and 27 young patients with secundum atrial septal defect (ASD) without overt left or right ventricular failure were compared. ASD patients were subdivided in low shunt ($Qp/Qs < 2.0$) and high shunt ($Qp/Qs \geq 2.0$) groups. Mean left ventricular (LV) stroke volume was significantly less in ASD patients ($46 \pm 16 ml/m^2$ in the low shunt and 44 ± 9 ml/m² in high shunt group) compared with control patients (51 ± 13 ml/m², $P < 0.01$ and $P < 0.02$, respectively). There was no significant difference in mean left ventricular end-diastolic volume (LVDEDV) between any group of patients (control subjects 67 ± 17 ml/m²; low shunt ASD 66 ± 17 ml/m², and high shunt ASD 62 ± 12 ml/m²). High shunt ASD had a significantly lower cardiac index compared with control patients (5.0 liters/min/m² vs. 5.9 liters/min/m², $P < 0.02$). Both low shunt and high shunt ASD showed significantly lower stroke work indices than control subjects (42 ± 13 GmM/m² and 37 ± 8 GmM/m² compared with 51 ± 14 GmM/m², $P < 0.05$ and $P < 0.001$, respectively) but only the high shunt group had a significantly lower peak systolic pressure (94 ± 12 mm Hg vs. 109 ± 11 mm Hg for control patients, $P < 0.01$). There was no significant difference between the control and ASD groups in LV end-diastolic, mean right atrial, right ventricular end-diastolic, and pulmonary pressures.

External systolic time intervals were compared in 5 control and 12 ASD patients. There was no significant difference between the two groups of patients in absolute values

or indices for pre-ejection period, ejection time, or electromechanical systole. However, the ratio of the pre-ejection period index to left ventricular ejection time index (PEPI/LVETI) was significantly higher in ASD patients ($P < 0.05$).

In young subjects with large shunt ASD, certain indicators of left ventricular function are depressed. Evaluation of PEPI/LVETI may allow noninvasive determination of LV function.

Hematology

Cole ER: *Alteration of the kinetics of thrombin-catalyzed hydrolysis of amino acid ester substrates by sodium cholate and other steroids. Thrombos Diathes haemorrh (Stuttg.) 32:132, 1974*

Thrombin-catalyzed hydrolysis of TAME proceeds by an initial zero-order phase which later falls off into an apparent first order reaction as substrate become limiting. Optimum amounts of sodium cholate not only accelerated TAME hydrolysis but also altered its kinetics to apparent zero-order to complete substrate hydrolysis. As this implies, the rate of hydrolysis in the presence of cholate was found to be independent of substrate concentration, provided concentrations of TAME and cholate were low enough to prevent precipitation of some TAME-cholate as an insoluble complex. The formation of a soluble complex composed of polymeric molecules of TAME and cholate may explain both the acceleration and the change in reaction order. Although the pH and temperature optima for TAME hydrolysis by thrombin were not altered by the presence of cholate, the degree of acceleration increased with rising pH and temperature on the ascending portion of the curves. This is believed to be due to the greater solubility of the TAME-cholate complex. The effects of cholate on thrombin-catalyzed hydrolysis of other arginine esters as well as esters of lysine, histidine and phenylalanine were also studied.

Solutions of sodium desoxycholate and androsterone-3-sulfate accelerated TAME hydrolysis as did suspensions of testosterone, etiocholanolone, androsterone, androsterone-3-hemisuccinate and pregnandiol, estradiol, estrone, estriol, estrone-3-sulfate, cholesterol. corticosterone, hydrocortisone and hydrocortisone-3-phosphate had significant effect on TAME hydrolysis by thrombin. The ability of the androgenic hormones to accelerate hydrolysis appeared to depend to some extent on the configuration of the substituent group at C_3 and the hydrogen at C_5. Androsterone-3-hemisuccinate was, like cholate, able to accelerate the hydrolysis of TAME at apparent zero-order kinetics to complete substrate hydrolysis.

McKenna R, Miró-Quesada M, Bachmann F: *Incidence of thromboembolism in patients with abnormally short activated partial thromboplastin time (APTT). Clin Res 21:832, 1973*

In a six-month period we performed 4195 APTT's as part of a coagulation screening profile. Of these, 71 percent were in the normal range (29-45 sec, mean 37, SD 3.2). Twelve percent of all APTT's were shorter than 29 sec (mean-2.5 SD). A prospective study was undertaken to determine the frequency of various disease states associated with short APTT's and the subsequent incidence of thromboembolism in 100 patients. The largest group (32 percent) consisted of patients with malignancies (2 percent had localized lesion, 30 percent had metastatic disease). The second group comprised severe peripheral vascular disease and/or coronary artery disease (20 percent). Chronic renal failure, diabetes, and collagen vascular diseases accounted for 21 percent.

Eighteen percent of the non-cancer and 23 percent of the cancer patients developed thrombophlebitis and/or pulmonary embolism. The incidence was slightly higher in patients with APTT's < 25 sec (21 percent and 27 percent, respectively). The median time for the development of thromboembolism was 7.5 days in both groups. The incidence of thromboembolism in patients with APTT's of < 29 sec is significantly higher than the overall incidence of clinically diagnosed thromboembolism at the hospital. Low dosage heparin therapy may be indicated in such patients as a prophylactic measure.

McKenna R, Whittaker B, Bachmann F: *Platelet function studies in patients undergoing open heart surgery (OHS). Proc Am Soc Hematol, Chicago, 1973, p. 118*

A survey of abnormal bleeding in patients after OHS revealed: i) a local bleeder in 75 percent, ii) intrinsic coagulation defects in 10 percent, and iii) essentially normal coagulation without severely reduced platelet counts in 15 percent, prompting a prospective study of platelet function in 13 patients undergoing single valve replacement or coronary artery bypass. Patients were evaluated (A) after premedication before anesthesia, (B) at the end of bypass, and (C) two hours after administration of protamine sulfate. From A to B the following changes were observed: reduction of platelet count ($p < 0.001$), of platelet adhesiveness ($p < 0.001$) and of fibrinogen by Ratnoff Menzie assay ($p < 0.02$); increase of fibrin split products (FSP) by the Merskey technique ($p < 0.01$); platelet aggregation in response to collagen, epinephrine and ADP and platelet factor 3 availability (PF3) assayed in standardized platelet-rich plasma (SPRP) containing 10^5 platelets/mm^3 were decreased. Between A and C a significant prolongation of the template bleeding time (TBT) occurred ($p < 0.001$). From B to C fibrinogen increased ($p < 0.02$) and FSP's fell ($p < 0.01$); the platelet counts rose slightly ($p < 0.03$) but adhesiveness and aggregation did not change significantly. In summary, after OHS with extracorporeal circulation a significant hemostatic defect was observed in eleven out of thirteen patients with marked prolongation of the TBT. This defect is only partially explained by the decreased platelet count. The abnormalities of platelet aggregation and PF3 measured in SPRP are independent of the platelet count and suggest the development of a qualitative platelet function defect during OHS, which is probably not caused by the increased level of FSP's. We, therefore, recommend that a platelet count and a TBT be determined in all patients with excessive bleeding after OHS to assess thrombocytopenia and platelet functional defects.

Microbiology

Deinhardt F, Wolfe L, Peterson D, Cross GF, Holmes AW: *The mythology of various hepatitis A virus isolates. Develop Biol Standard 30:390, 1975*

Several types of viral hepatitis may exist. Hepatitis A (MS-1 type) can be transmitted to marmosets and chimpanzees. Virus-like particles, which may be parvo- or enteroviruses and which have been demonstrated in feces of this type of hepatitis, do not share cross-reacting antigens with hepatitis B but do cross-react with fecal hepatitis A antigen. Hepatitis A (GB type), which also does not cross-react with hepatitis B, is not antigenically identical with MS-1; it can be transmitted to marmosets and it may be similar to non-type A/non-type B post-transfusion hepatitis.

Hepatitis B does not cross-react either with HA particles, the faecal hepatitis type A antigen or with the MS-1 or GB strains; it can be transmitted to chimpanzees and rhesus monkeys but not to marmosets.

Medical Education

Hejna WF: *It takes four years . . . Reversing the acceleration trend. JAMA 234:387, 1975*

Many of the arguments advanced in favor of shortening the standard medical school curriculum to three years may no longer be valid, and such shortening may cause tensions among students and faculty. Further, this innovation in curriculum does not materially address issues such as physician availability, quality, and maldistribution. Success in curricular design is intimately related to accommodation of individual intellectual pursuit, cultivation of independent thinking, and redefinition of core material. Such aspects developed on a sound biomedical scientific base will best serve future physicians regardless of career choice, and are best presented in a four-year time frame.

Neurology

Fox JH, Patel-Mandlik K, Cohen MM: *Comparative effects of organic and inorganic mercury on brain slice respiration and metabolism. J Neurochem 24:757, 1975*

Respiration and carbohydrate metabolism were measured in guinea-pig brain slices exposed to organic and inorganic mercury. Organic mercury decreased oxygen uptake and $^{14}CO_2$ production at consistently lower concentrations than inorganic mercury. Organic mercury also caused a striking elevation of pyruvate and lactate at low doses where inorganic mercury had no effect on respiration or metabolism. A unique inhibition of tricarboxylic acid cycle function is suggested and might partially explain the distinctive neurotoxicity of organic mercury.

Garvin JS: *The electroencephalogram in hereditary spino-cerebellar degenerations. Clin Electroencephalog 5:19, 1974*

The differential diagnosis of various types of spino-cerebellar degeneration is difficult, and outstanding experts often disagree. Abnormal electroencephalograms are present in a high percentage of cases. The present study raises the possibility that differences in the electroencephalographic findings will aid in a more precise diagnosis of these disorders and in a more accurate prognosis. More cases are needed to determine the degree to which the electroencephalographic findings will assist in the classification of cases of spino-cerebellar degeneration, but if clinicians and electroencephalographers are aware that electroencephalogram studies may be of assistance, (instead of assuming as is generally the case at present, that cerebellar disease produces no useful electroencephalogram finding), a sufficient number of cases will doubtless soon be accumulated.

Garvin JS, Gibbs EL: *Electroencephalogram in hydrocephalus. Clin Electroencephalog 6:29, 1975*

In our series of 320 hydrocephalics we have found the most common electroencephalographic abnormality to be asynchronous sleep patterns (66.8 percent). The next most common abnormality was seizure activity (60.3 percent), yet only 38 percent had clinical seizures. The type of electroencephalographic patterns were essentially the same in all cases regardless of etiology. Those cases with spontaneous arrest had the highest percentage of normal electroencephalograms and the lowest percentage of institutionaliza-

tion, yet 71 percent were mentally retarded. There was a slightly higher percentage of seizure activity and slow wave foci in patients with shunts than those not shunted, yet only 64 percent with shunts were retarded compared to 80 percent without shunts. Of the shunted group 23 percent were institutionalized while 36 percent of those without shunts were institutionalized. It would appear that although there is little improvement in the electroencephalogram pattern of patients with hydrocephalus who have shunt procedures, the decrease in percentage of retardation and institutionalization often make this procedure worthwhile.

Nuclear Medicine

Fordham EW, Ramachandran PC: *Radionuclide imaging of osseous trauma. Semin Nucl Med 4:411, 1974*

Radionuclide imaging in osseous trauma is now possible with the availability of low-radiation dose bone-localizing agents. Bone trauma results in increased uptake of activity at the traumatic site by the third day. Maximum uptake occurs at three to five weeks and may resolve over a variable time. Increased uptake at the site of a fracture may persist for many years. Osseous imaging has proved useful in neurologic problems, circulatory disease, aseptic necrosis, direct bone trauma, stress, iatrogenic trauma, and radiation injury. The agents of choice currently are ^{18}F and the bone-localizing compounds of $^{99m}T_c$.

Oncology

Piel IJ, Meyer D, Perlia CP, Wolfe VI: *Effects of Cis-diamminedichloroplatinum (NSC-119875) on hearing function in man. Cancer Chemother Rep 58:871, 1974*

Fifty-four serial audiograms performed in 30 patients who had received cis-diamminedichloroplatinum were analyzed. Four patients demonstrated significant hearing loss at 2000 Hertz (Hz), 14 at 4000 Hz, and 18 at 8000 Hz. The incidence of ototoxicity increased with increasing doses of the drug. In the presence of renal toxicity, hyperuricemia, or bone marrow depression, decrease of pure-tone threshold at both 4000 and 8000 Hz was more common. Speech-discrimination thresholds showed no consistent pattern of relationships. The possible etiology and pathogenesis of ototoxicity are discussed.

Orthopedic Surgery

Escalas F, Galante J, Rostoker W, Coogan PS: *$MP_{35}N$: a corrosion-resistant, high-strength alloy for orthopedic surgical implants: bio-assay results. J Biomed Mater Res 9:303, 1975*

A cobalt-based alloy, $MP_{35}N$, with excellent mechanical properties has been recently introduced as a material for surgical orthopedic implants.

A study was made of local and systemic host response to this material in two different mammal species. The implantation time ranged from one to 12 months.

The result of this study indicated: $MP_{35}N$ produces a degree of local tissue response comparable to that of 316L stainless steel. No systemic side effects were observed during the implantation times included in this study.

Galante JO, Rostoker W, Doyle JM: *Failed femoral stems in total hip prostheses: a report of six cases. J Bone Joint Surg 57:230, 1975*

Six femoral stems of total joint prostheses failed and were studied. These included two short-neck Muller, one standard Muller, one new design long-neck Muller, and two Charnley prostheses. In addition, reference is made to another failed Charnley prosthesis which had not required revision at the time of the study. Reoperation and replacement of the femoral component was required in all six cases. Metal fatigue appeared to be the cause of failure in all instances. Metallographic examination of the removed prosthesis revealed no underlying defects in one prosthesis. In the other five prostheses metallurgical defects were found. Varus placement of the femoral prosthesis or loosening of the cement-prosthesis bond, or both, were identified in five of the six and were thought to have led to overload. A combination of metal defects and loosening or malpositioning was thought to be responsible for the failures. It was further indicated that current designs should be regarded as marginal in relation to long service life, until more fatigue information is available on the metallic materials currently in use.

Rostoker W, Pretzel CW: *Couple corrosion among alloys for skeletal prostheses. J Biomed Mater Res 8:407, 1974*

Using a metallographic technique to identify and count corrosion pits, the tendency for passive film breakdown has been studied under conditions that simulate a crevice and a combination of two different materials. Potential corrosion couples were formed among: 316L stainless steel, cast Co-Cr-Mo alloy, wrought Co-Cr-W-Ni alloy, Ti-6 percent Al-4 percent V alloy, a Co-Ni-Cr-Mo (multiphase) alloy and graphite. Only 316L stainless steel seems significantly prone to pitting corrosion in a crevice condition. Dissimilar metals in the crevice configuration did not seem to accelerate the corrosion of stainless steel.

Tetik RD, Galante JO, Rostoker W: *A wear-resistant material for total joint replacement—tissue biocompatibility of an ultra-high molecular weight (UHMW) polyethylene-graphite composite. J Biomed Mater Res 8:231, 1974*

A new prosthetic material, ultra-high molecular weight (UHMW) polyethylene graphite, was developed for use in total joint replacement and found to exhibit 1/7 to 1/30 lower wear rate than UHMW polyethylene bearing against Vitallium alloy.

A preliminary study was undertaken to test the biocompatibility of the new material. Specimens of 316L stainless steel, high-purity graphite, UHMW polyethylene, and the composite material were implanted in rabbits and cynomologous monkeys. The materials were evaluated in both solid and particulate forms after implantation in bone, joint cavities, and paravertebral muscles. The tissue reactions to the materials were noted at time intervals from six weeks to one year.

All materials showed good tissue acceptance in solid form. The UHMW polyethylene and the composite material were noted to be more reactive than either the high-purity graphite alone or the stainless steel when in particulate form.

The eventual use of the polyethylene graphite composite in total hip arthroplasties is suggested by virtue of its identical biocompatibility with UHMW polyethylene. In addition, its superior wear resistance, and hence low volume of wear debris particles presented to the surrounding tissues would offer an advantage in terms of longevity of the implant both mechanically and biologically.

Hegyvary C: *Covalent labeling of the digitalis-binding component of plasma membranes. Mol Pharmacol 11:588, 1975*

Oxidized [^{3}H] ouabain was prepared and its binding to renal plasma membranes was studied. More than 90 percent of the oxidized ouabain was bound by the ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) component of the membranes. This binding required the simultaneous presence of Na^+, Mg^{++}, and ATP. K^+ inhibited binding. No binding took place in the absence of Mg^{++}. Oxidized [^{3}H] ouabain was covalently attached to the membranes by reducing the ouabain-membrane complex with $NaBH_4$ at pH 9.0. The covalent nature of this binding was evident from its stability against dissociation by acid, 90 percent methanol, or heating. In the presence of Na^+, ATP, and Mg^{++} oxidized [^{3}H] ouabain was bound preferentially by an electrophoretically single membrane protein. This protein had an apparent molecular weight of 89,000 as estimated by polyacrylamide gel electrophoresis, and it is thought to be part of the ($Na^+ + K^+$)-ATPase because (a) it bound ouabain in the presence of Na^+, Mg^{++}, and ATP but not in the presence of Tris-EDTA, and (b) it could be phosphorylated by [^{32}P] ATP in the presence of Na^+ and Mg^{++} but not in the presence of K^+ and Mg^{++}.

Rieckmann KH, Trenholme GM, Williams RL, Carson PE, Frischer H, Desjardins RE: *Prophylactic activity of mefloquine hydrochloride (WR 142490) in drug-resistant malaria. Bull World Health Organ 51:375, 1974*

In preliminary studies with mefloquine (WR 142490) a single dose exerted prolonged suppressive activity against a drug-resistant strain of *Plasmodium falciparum.* Development of patent parasitaemia was prevented when nonimmune persons were exposed to infected mosquitos two weeks after medication, and it was delayed when exposure occurred three weeks after drug administration.

Trenholme GM, Williams RL, Patterson EC, Frischer H, Carson PE, Rieckmann KH: *A method for the determination of amodiaquine. Bull World Health Organ 51:431, 1974*

A new fluorometric method for analysis of amodiaquine in serum, plasma, or red cells is described. Amodiaquine is extracted from alkalinized biological fluid into 1,2-dichloroethane and is then re-extracted into 0.1 N hydrochloric acid. Borate buffer is added to the acid solution and the resultant solution is heated for 30 minutes in boiling water. Heating the buffered solution produces a marked increase in the fluorescence of amodiaquine, which may then be measured. Standard curves prepared in serum and red cells were linear between 50 and 3000 μg/litre. Reproducibility of the assay and recovery of amodiaquine from serum and red cells were satisfactory. The specificity of the assay and the nature of the induced fluorophor are not known. The paper indicates representative serum and red cell levels of amodiaquine after the administration to five subjects of 10 mg of amodiaquine base per kg of body weight.

Williams RL, Trenholme GM, Carson PE, Frischer H. Reickmann KH: *Acetylator phenotype and response of individuals infected with a chloroquine-resistant strain of* Plasmodium falciparum *to sulfalene and pyrimethamine. J Trop Med Hyg 24:734, 1975*

Acetylator phenotype was determined in 33 volunteers who were infected with a chloroquine-resistant strain of *Plasmodium falciparum* and who received, for cure 2 g of sulfa-

lene and 50 mg of pyrimethamine. This drug combination did not cure 5 of 14 rapid acetylators and 3 of 19 slow acetylators. This difference is not significant. Plasma levels of non-acetylated sulfalene, acetylated sulfalene, percentage acetylation, and biologic half-life of non-acetylated sulfalene after administration of the combination did not differ importantly between the two groups. Acetylator phenotype does not appear to influence the response to sulfalene and pyrimethamine of individuals infected with chloroquine-resistant falciparum malaria.

Physiology

Hegyvary C: *Effects of some organic solvents on the reactivity of sodium* plus *potassium ion-transport ATPase. Biochim Biophys Acta 311:272, 1973*

1. Organic solvents including aliphatic alcohols, acetone, diethyl ether and chloroform inhibited ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) of the plasma membranes of guinea pig kidney. Inhibition was uncompetitive with respect to ATP if the ATP concentration was larger than 5 μM. Below this concentration of ATP, K^+ inhibits rather than stimulates the Na^+-dependent splitting of ATP by the native enzyme. The tested organic solvents converted this K^+ inhibition into K^+ stimulation. In the presence of these solvents, the apparent affinity of the enzyme increased for Na^+ and ATP and decreased for K^+ as estimated by the kinetics of ATP hydrolysis. Apparent homotropic allosteric interaction between the Na^+ and K^+ sites increased.

2. Organic solvents interfered with different steps of the transient phosphorylation of the enzyme. As estimated by pulse labeling, organic solvents enhanced interaction of ATP with the enzyme prior to phosphorylation both in the absence or in the presence of K^+. The solvents decreased the rate of dephosphorylation of the phosphoenzyme either in the presence of K^+ (native enzyme) or in the presence of ADP (*N*-ethylmaleimide-treated enzyme) but they did not affect the ratio of the ADP-sensitive form of the native phospho-enzyme to the K^+-sensitive form. Rephosphorylation by ATP of the dephospho-enzyme, formed by adding Rb^+, was accelerated by organic solvents.

3. Interaction of the enzyme with these organic solvents appeared to be primarily hydrophobic, as the half-maximal inhibitory concentrations of these solvents correlated with their octanol-water partition coefficients and with the length of the hydrophobic side chain in the series of homologous aliphatic alcohols.

4. It is considered that these modifications of ($Na^+ + K^+$)-ATPase were due partly to conformational changes of the enzyme, and partly to changes in the water structure around the active center which is proposed to be in a hydrophobic environment.

Psychiatry

Berger JC: *Traffic accidents, facial injuries, and psychiatry. Clinics Plast Surg 2:3, 1975*

The extent to which emotional factors play a direct or indirect role in the causation of traffic accidents has been presented along with the early and late emotional response of individuals to facial injuries as a result of traffic accidents. Illustrated case histories are presented.

West JW: *Alcoholism—a general hospital meets the challenge. Ill Med J 146:99, 1974*

This care of alcoholism will conform to the standards of such care set by the Joint Commission on Accreditation of Hospitals. The two components of care which this, and any

other general hospital can provide, are emergency care and aftercare.

Some of the more elaborate psychosocial group therapeutics at Little Company of Mary Hospital would not be necessary for all general hospital programs. Local volunteer A.A. people from the community can provide much good counseling, and many hospitals now have A.A. groups which meet in the hospital area. Ideally, every general hospital should take care of the acute alcoholism patient in the community, and establish an aftercare system which would include an alcoholism rehabilitation center to which patients, who require more than a short inpatient epperience, could be referred. A good rehabilitation center can serve a constellation of referring general hospitals. General hospitals would then be providers of acute care for which most of them have been designed.

Radiology

Kaszniak AW, Garron DC, Fox JH, Huckman MS, Ramsey RG: *Relation between dementia and cerebral atrophy as measured by computerized tomography. Neurology 25:387, 1975*

Computerized tomography (CT Scan) is a new method for visualizing cerebral structures, including the ventricles and corticol sulci. Cerebral atrophy, derived by combining measures of ventricular size and enlarged sulci, was determined in 31 patients aged 47 to 84 suspected of dementia, but without evidence of acute focal lesion. Patients were also given eight cognitive and mnemonic tasks. Data were subjected to a stepwise, multiple regression analysis. This analysis calculates the variance attributable to the relation between the dependent variable (cerebral atrophy) and each of the other variables (cognitive and mnemonic tasks), and determines both the statistical significance and the relative value of each of the tasks as a predictor of cerebral atrophy. Significant predictors, in decreasing order of value, are a) memory for pictured common objects, b) understanding of incongruous pictured situations, and c) use of common implements with the left hand. These results indicate that increasing degrees of general atrophy, as measured by computerized tomography, are associated with increasing difficulty in a) immediate memory, b) social judgment, and c) performing familiar manual tasks with the unaccustomed hand. These findings also enhance the value of computerized tomography in evaluating patients with dementia.

RUSH-PRESBYTERIAN-ST. LUKE'S

MEDICAL BULLETIN

VOL. 15, NO. 2 APRIL, 1976

Perspectives in Viral Hepatitis

RICHARD B. CAPPS SYMPOSIUM

Rush-Presbyterian-St. Luke's Medical Bulletin is published quarterly by the staff of Presbyterian-St. Luke's Hospital, Rush Medical College, and the Alumni Foundation.

All correspondence relative to the publication of papers should be addressed to the Editor, Rush-Presbyterian-St. Luke's Medical Bulletin, Room 242, 1725 West Harrison Street, Chicago, Illinois 60612. All other correspondence should be addressed to Rush-Presbyterian-St. Luke's Medical Bulletin, Room 1007, 1725 West Harrison Street, Chicago, Illinois 60612. Requests for reprints should be sent direct to the authors.

VOL. 15 NO. 2 APRIL 1976

TABLE OF CONTENTS

RICHARD B. CAPPS SYMPOSIUM

Address of Welcome

(Presented by James A. Campbell, M.D., President of Rush-Presbyterian-St. Luke's Medical Center, at the Richard B. Capps Symposium on Perspectives in Viral Hepatitis, March 18, 1976, at Chicago, Illinois)

Welcome to the Richard B. Capps Symposium on Perspectives in Viral Hepatitis. All of us have convened today in order to say to Dick, "Happy Seventieth Birthday."

This occasion gives each one of us, particularly me, the opportunity to acknowledge publicly to Richard our deeply held personal affection and respect for him as a scientist, physician, person, and friend. There are few persons at Rush-Presbyterian-St. Luke's who could be honored for such an easy, natural combination of breadth of interests and distinction of achievement.

Some of us have known Dick since his role as one of the "giants of the Thorndike," all outstanding clinical investigators. Following his graduation from the Harvard Medical School in 1931, and after he had served as a house officer at the Massachusetts General Hospital, his avid scientific curiosity was drawn to the complexities of the pressures within the cardiovascular system. He became a member of the Thorndike Lab; and, with Soma Weiss and Gene Ferris, elucidated mechanisms of syncope associated with the hypersensitive carotid body. These studies quite naturally for one of Richard's drive and imagination expanded to encompass research in vasomotor tone, hypertension, and peripheral vascular disease.

He came home to Chicago in 1936 where he continued his effectiveness as a "triple threat" clinician, investigator, and teacher, with special interest in vascular disorders until World War II when circumstances, of which all of us here today are well aware, drew his talents to the problem of acute infectious hepatitis.

His initial wartime observations, and those he made later under somewhat less hectic circumstances, have laid the foundations of modern hepatology upon which many of the findings reported here today have been built.

The research interest and clinic skills of Dick Capps inevitably have attracted many patients, students, and colleagues. He is now a "giant at Rush-Presbyertian-St. Lukes's." His staff colleagues here have asked him, not once, but twice to serve as their President. His research fellows and residents revere him, as their presence here on his birthday attests.

Today, we know him as a contemporary, sophisticated clinical investigator. I want to add, however, that his patients recognize him as a "doctor of the old school." He cares for patients with the understanding and in the tradition of Francis W. Peabody and Joseph B. Capps. This devotion to his patients is exceeded only by their devotion to him. He has been honored by them through their establishment here at Rush-Presbyterian-St. Luke's of the Richard B. Capps Chair in Hepatology, and, of course, by their assistance in making this symposium possible.

On behalf of all of your patients, Richard, your colleagues, students, and friends, thank you for what you have done on our behalf. May you continue your efforts with the enthusiasm and success that have marked your first seventy years, and may we say again, "Happy Birthday!"

Foreword to the Symposium

On March 13th, 1976, clinicians and laboratory scientists, students and research fellows gathered in the A.B. Dick Lecture Theatre at Rush-Presbyterian-St. Luke's Medical Center to honour both the seventieth birthday of Richard B. Capps and his contributions to hepatology.

The subject of viral hepatitis was chosen for the symposium because Dr. Capps' own studies laid some of the foundations for this clinically important field, and, in recent years, advances in understanding viral hepatitis have been rapid and multifaceted. An examination of the perspectives, past, present and future, seemed timely, and the program was divided into two parts, the laboratory aspects of viral hepatitis A and B, and the clinical aspects of viral hepatitis. Professor Zuckerman summarized the current situation in viral hepatitis, relating historical developments to our present state of knowledge. Drs. Overby, Dienstag and Hollinger spoke of work in progress in their laboratories, which will lead to considerable advances and improvements in our ability to diagnose infections with the viruses of hepatitis A and B, and to characterize the infectious agents. During the clinical session Dr. Leevy discussed criteria for diagnosing and treating viral hepatitis, Dr. Schaffner spoke about morphology, and Dr. Summerskill about the mechanisms and management of chronic hepatitis.* The meeting closed with Dr. Williams' report on fulminant hepatitis and a roundtable discussion.

This issue of the Rush-Presbyterian-St. Luke's Medical Center Bulletin contains the papers presented at the meeting, and is intended as a *Festschrift* to honour Dr. Richard B. Capps. On his behalf I thank the friends of Dr. and Mrs. Richard B. Capps and Abbott Laboratories, North Chicago whose support made the symposium possible. I also thank the speakers for their excellent papers and stimulating discussion, and the chairmen (Drs. Robert M. Kark, Rush-Presbyterian-St. Luke's Medical Center, Chicago; Dr. Hans Popper, Mount Sinai School of Medicine, New York; Dr. A. W. Holmes, Texas Tech University School of Medicine, Lubbock, Texas) and my wife, Dr. Jean Deinhardt, for their help in making the symposium such a success.

FRIEDRICH DEINHARDT

Friedrich Deinhardt, M.D., Professor at Rush Medical College and College of Health Sciences, is Chairman of the Department of Microbiology at Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois

*Dr. William H. J. Summerskill, of the Mayo Foundation, was unable to submit the manuscript of his presentation for publication.

TWENTY-FIVE CENTURIES OF VIRAL HEPATITIS

Arie J. Zuckerman

In the beginning . . . . .

Descriptions of disease of the liver, particularly jaundice, are to be found in the Babylonian Talmud (5th Century B.C.), and jaundice appears to have been common at the time. Hippocrates, during this period, described epidemic jaundice as the "fourth kind of jaundice" but the interpretation of the term "epidemic" is in doubt, since yellow bile was regarded as one of the four humours and the agent responsible for most fevers. It seems that the contagious nature of jaundice was first mentioned in the 8th century A.D. in a letter from Pope Zacharias to St. Boniface, Archbishop of Mainz (Cockayne[1]). Pope Zacharias urged that patients with jaundice should be separated, lest others catch the contagion. The first definite description of an epidemic of jaundice among civilians was mentioned by Herlitz in Gottingen in 1791. Although Herlitz introduced the term "icterus epidemicus," Sydenham in London (1624-1689) had already recorded detailed observations of epidemic jaundice. The concept of epidemic jaundice, however, was not accepted.

Arie J. Zuckerman, M.D., Professor of Microbiology, University of London; Director of the World Health Organization Collaborating Centre for Reference and Research on Viral Hepatitis; Department of Microbiology, London School of Hygiene and Tropical Medicine, London, WC1E 7HT, England

The view that catarrhal jaundice was obstructive and not hepatic in origin was first expressed by Bamberger[2] in 1855, who considered that swelling of the ostium of the common bile duct was the principal cause. This view is commonly ascribed to Virchow,[3] who described, in 1865, the pathology of hepatitis after examination of a single case in which the terminal portion of the common bile duct was plugged by mucus from the duodenum. Onset of the disease was always associated with a gastrointestinal upset, and it was assumed that a microbial infection, not necessarily specific, spread upwards from the intestine to block the bile duct by catarrhal inflammation or cholangitis, and thus the term "catarrhal jaundice" was introduced. Frohlich,[4] when reviewing 30 outbreaks of jaundice in 1879, reported a suggestion that an infectious process might be implicated in only one outbreak. This fitted well with the accepted view that all varieties of jaundice were essentially obstructive. Eppinger[5] reported in 1908 the case history of a girl, aged 19, who fractured her skull and died as the result of jumping out a window the day following her admission to hospital for treatment of a typical attack of catarrhal jaundice. At autopsy the liver was found to be normal macroscopically and on histological examination, but the mucous membrane of the stomach and duodenum was swollen, and the papilla of Vater was prominent. The ostium of the common bile duct was blocked as a result of inflammation of its wall and

hypertrophy of lymphoid tissue. Eppinger[6] taught that all jaundice was obstructive in origin, whether obstruction occurred in the larger extrahepatic ducts, as in catarrhal jaundice, or in the biliary capillaries, as in cirrhosis. This is surprising since it was widely known that epidemic jaundice occurred during the Middle Ages, particularly during wars ("campaign jaundice"), and jaundice closely followed in importance plague and cholera as the cause of pandemics in Europe. The excellent historical account of epidemic jaundice by von Bormann and his associates[7] refers to an outbreak in Germany in 1629, and they also mentioned an outbreak in the British Army in Flanders in 1743. In 1764, Monro[8] carefully documented jaundice in his account of diseases common in British troops in Germany from January, 1761 to March 1763.

Jaundice plagued Napoleon's army in Egypt but it is uncertain whether this outbreak was due to infective hepatitis because of the high mortality rate. During the American Civil War (1861-1865) 71,691 cases of jaundice were reported in the Union Army. Epidemic jaundice occurred during the Franco-Prussian war in 1870 among the troops and among the civilian population during the siege of Paris. The French referred to infective hepatitis as *jaunisse des camps,* and the Germans as *Soldatengelbsucht*. During the Boer War in South Africa 5648 cases of jaundice were recorded, and the mortality was low. Large epidemics of jaundice occurred in the Japanese navy during the war with Russia (1904-1905), and huge epidemics of hepatitis were recorded during the First World War, particularly in the Middle East theatres.

Epidemics of infective hepatitis during the Second World War attained vast proportions; over 5,000,000 cases occurred in the German armies and civilians alone (Gutzeit[9]), whilst huge epidemics swept through the Allied Forces especially in the Mediterranean region (Cullinan[10]). Indeed the number of cases was so large as to influence the strategy of the war. Despite the tremendous advances in the knowledge of the epidemiology of hepatitis, this infection was again a serious problem in the Israel War of Independence in 1948, the Arab-Israel conflicts in 1956 and 1967, during the Korean campaigns, and in South Vietnam.

The view that catarrhal jaundice was not due to mechanical obstruction of the extrahepatic biliary tract, despite the presence of inflammation of the upper gastrointestinal tract was first promulgated in 1839 by Stokes.[11] This was subsequently supported by the work of Heitler[12] in 1887 and Flindt[13] who considered, in 1890, that this was a generalized infection which reached the liver parenchyma through the bloodstream. It was also concluded that hepatitis might occur in both sporadic and epidemic form and that it was intimately associated with acute and subacute necrosis of the liver. Similar observations were reported by Martin[14] in 1918 on cases of jaundice occurring during the Gallipoli campaign. Necrosis of the parenchymal cells of the liver was found, and it was considered that the jaundice was due to hepatitis following a systemic infection rather than to catarrhal jaundice resulting from obstruction of the bile ducts.

The awakening . . . . .

McDonald[15,16] in 1908 and again in 1918 first predicted that infective jaundice was probably caused by an agent smaller than a bacterium and postulated that it was a virus. The viral etiology of infective hepatitis gained support from then onwards (Stokes, Ruedemann and Lem-

on;[17] Bergstrand;[18] Findlay, Dunlop and Brown;[19] Findlay and Dunlop[20]). The etiology of infective hepatitis was reviewed by Findlay, MacCallum and Murgatroyd[21] who pointed out that in the absence of a laboratory animal which can be infected with hepatitis, the viral etiology of the disease would have remained hypothetical had it not been for the occurrence of hepatitis following immunization against yellow fever. Human volunteer studies during the Second World War and subsequently finally established the viral etiology of infective hepatitis.

The history of serum hepatitis is much shorter. Lurman[22] reported in 1885 the earliest recognized epidemic of serum hepatitis among shipyard workers in Bremen in 1883. Some cases of smallpox occurred in Bremen and extensive vaccinations were carried out with glycerinated lymph of human origin. Of 1289 vaccinated employees 191 developed jaundice after intervals of several weeks to six months. Several hundred workers employed after the vaccination had been completed, and those inoculated with different batches of "lymph," were not affected. Lurman's paper is a classic example of meticulous epidemiological observations.

MacCallum[23] provided excellent and fascinating historical perspectives on viral hepatitis, including his many personal recollections. Reference will be made to this account later; and, as McCallum pointed out, it is strange that no record of other events such as those described by Lurman had been found earlier in view of the practice of using human lymph for smallpox vaccination and the widespread practice of tattooing. The wide-scale introduction and common use of large syringes and long needles with the advent in 1909 of salvarsan therapy in venereal disease clinics was soon followed by sudden outbreaks of jaundice. The drug was naturally suspected as a potential hepatotoxin. The Salvarsan Committee of the Medical Research Council[24] which published two reports in 1919 and in 1922, could come to no decision on the precise cause of jaundice, but toxicity of special batches of the drug was excluded. Of special interest in 1917 was an outbreak of jaundice with 15 deaths at the venereal disease department of Cheryhinton Military Hospital, Cambridge, and the MRC committee drew attention to the fact that at the same time there was a small epidemic of jaundice among children in an elementary school nearby, affecting 15 children and one adult. Outbreaks of jaundice were later again reported in venereal disease clinics. Murray[25] noted in 1930 that jaundice occurred in 11 percent of 118 soldiers 60 to 126 days after intravenous injection with acriflavine for the treatment of gonorrhea. Of interest is that human serum was added to acriflavine. It was thought that the effectiveness of the drug was enhanced by the added serum. Ruge[26] observed in 1932 that among 2459 patients with jaundice in the German navy between 1919 and 1929, 700 cases followed treatment with arsenic for syphilis. The jaundice was attributed to the hepatotoxicity of arsenic. Soffer[27] recorded in 1937 jaundice among patients treated with bismuth injections for syphilis, and ascribed this to the toxicity of bismuth and neoarsphenamine on the liver. In 1943 Bigger[28] drew attention to the rising incidence of jaundice among clinic patients receiving antisyphilitic treatment. The incidence of jaundice increased from 4.2 percent in 1941 to 16.5 percent the following year.

But outbreaks of jaundice were not restricted to venereal disease clinics. Flaum, Malmros and Persson[29] reported in 1926 epidemic jaundice in a diabetic clinic, and Sherwood[30] recorded in 1950 syringe-

transmitted hepatitis in four of nine patients with diabetes treated in a hospital. A common syringe was used for the administration of insulin. Droller[31] previously observed hepatitis among diabetics, but on that occasion it was traced to a common syringe used for the withdrawal of blood for blood sugar estimations. Hartfall, Garland and Goldie[32] reported in 1937 hepatitis indistinguishable from catarrhal jaundice in 85 of 900 patients treated for rheumatoid arthritis with injections of gold.

It was not until 1943 that a common factor was suggested in all these outbreaks. MacCallum[33] stated that jaundice may be transmitted from patient to patient by means of syringes which had been imperfectly sterilized between injections.

In 1938, MacNalty[34] reported the occurence of jaundice and a number of deaths in children in Oxford after injection of measles-convalescent serum from one batch. Propert[35] observed in the same year that a number of children in an institution developed hepatitis 60 days after the injection of human convalescent measles serum. Interestingly, other children, also 60 days later, who did not receive serum, developed hepatitis, and it seems possible that this was due to oral transmission, as was established later by Krugman and his associates. The term homologous serum jaundice came into use in Britain after publication of a Ministry of Health memorandum in 1943 describing an outbreak of 41 cases of jaundice and 8 deaths which followed subcutaneous injection of measles convalescent serum into children. Earlier in 1937, Findlay and MacCallum[36] drew attention to jaundice which followed yellow fever immunization, and they considered that the jaundice may have been due to some organism injected with the virus or serum. However, they reasoned that if a hypothetical virus pathogenic for man were injected directly with the inoculum it was surprising that under 3 percent of persons developed symptoms. Although the presence of a hypothetical virus could not be entirely excluded, the evidence against it was very great. Nevertheless, it was concluded that incidence of jaundice two to six months after yellow fever immunization was analogous to the occurrence of outbreaks following antisyphilitic treatment or injections of acriflavine.

Similarities were also pointed out between this form of hepatitis and the acute liver necrosis of horses, which was known as "staggers" in South Africa.

In October, 1939, about 27 percent of 304 persons inoculated with one lot of yellow fever vaccine developed jaundice four months after injection. In May, 1940, more cases of jaundice appeared in South America in relation to different lots of vaccine. Of 107,000 people, 1072 developed jaundice 12 to 20 weeks following injection with the incriminated batches of vaccine. However, the largest outbreak of serum hepatitis occurred in 1942 when 28,585 young American soldiers inoculated with yellow fever vaccine developed jaundice and 62 of them died.[37] There was considerable suggestive evidence that hepatitis and jaundice following yellow fever immunization was due to a filterable agent present in the human serum incorporated in the serum-Tyrode virus culture medium containing minced chick embryo (Findlay and MacCallum;[36] Findlay, MacCallum and Murgatroyd[21]). In 1939 it was concluded ". . . that pools of apparently normal human serum should not be used for inoculation unless the medical history of all donors can be followed over a considerable period of time, preferably at least one month, the probable incubation period of infective hepatitis."[21]

An incident which concerned F. O. MacCallum is best told in the original words[23] ". . . One day in 1942, I received a message to go to Whitehall to see one of the senior medical advisers and when I arrived I was asked, 'What is this yellow fever vaccine and how dangerous is it?' After explaining its constitution and the possibility of a mild reaction four to five days after inoculation I was told that the Cabinet was at that moment debating whether or not Mr. Churchill should be allowed to go to Moscow, which he wished to do in a few days' time. The yellow fever vaccine inoculation was theoretically essential before he could fly through the Middle East, but I explained that no antibody would be produced before 7 to 10 days so that there would be little point in giving the vaccine. It was finally decided that the vaccine would not be used, and the administrators would take care of the situation. Several months later, I received an irate call from the Director of Medical Services of the R.A.F., who had been inoculated from the same batch of vaccine which would have been used for Mr. Churchill, and was informed that the D.G. had spent a very mouldy Christmas with hepatitis about 66 days after his inoculation. This was the first I knew that we were in for trouble again with our vaccine after a lapse of five years. Unfortunately, owing to the war, I had never received the information that it had been found in Brazil that serum was not necessary for stabilization of the 17D virus in the vaccine. I will leave you to speculate on what might possibly have been the effect on the liver of our famous statesman and our ultimate fate if he had received the icterogenic vaccine".

Jaundice following the use of sandfly fever vaccine was reported in Russia in 1940 by Sergiev and associates,[38] following tranfusions with plasma or reconstituted dried human serum by Morgan and Williamson[39] in 1943, Spurling, Shone and Vaughan[40] in 1946, Brightman and Korns in 1947 and by others, following tranfusion with whole blood by Beeson[41] in 1943 and others, and following mumps-convalescent plasma by Beeson, Chesney and McFarlan[42] in 1944 and also by McFarlan and Chesney.[43] Transmission of the virus of hepatitis by blood transfusion thus became recognized, as well as the risk of hepatitis associated with the use of pooled and dried human plasma and human blood products (reviewed by Zuckerman[44,45] in 1970 and 1975).

The volunteer era

The viral etiology of hepatitis was finally established during the Second World War by successful experimental transmission to human volunteers, first in Germany in 1942 by Voegt,[46] in the British Mandate of Palestine by Cameron[47] in 1943, and later by more extensive studies which were carried out in Great Britain (reviewed by MacCallum *et. al.*[48]), and in the United States (reviewed by Havens[49,50]). Inevitably there are many limitations to this type of investigation; nevertheless the studies in volunteers have furnished considerable valuable information about the mode of transmission and the infectivity as well as some of the physical and chemical properities of the viruses causing hepatitis. Many of the early studies were carried out mainly with volunteers who were conscientious objectors to the war, prisoners, volunteer patients with rheumatoid arthritis, and mentally-handicapped children at the Willowbrook State School in New York.

During the early experimental work, it was difficult to make a clear distinction between infectious hepatitis and serum hepatitis in the absence of specific laboratory tests. Soon, however, differences between the two types became apparent

and these were based essentially on epidemiological observations, particularly the route of infection and the period of incubation. The terms *hepatitis A* for infectious or epidemic hepatitis, and *hepatitis B* for serum hepatitis or homologous serum jaundice were introduced by MacCallum[51] in 1947 and generally adopted in 1973 (World Health Organization[52]).

Additional information and confirmation of earlier results were obtained from the studies carried out since 1956 at the Willowbrook State School, New York, an institution for mentally retarded children. The patient population at this institution increased from 200 children in 1949, to over 6,000 in 1963, and viral hepatitis has been an endemic disease among the children since 1953. Since 1956, 1,153 cases of viral hepatitis with jaundice were observed to have been transmitted by natural contact in this institution. Most of the newly admitted children contract the infection within the first 6 to 12 months of admission. During the last 12 years, approximately 250 children participated in experimentally induced hepatitis at Willowbrook, and these studies have been conducted in accordance with the World Medical Association's Draft Code of Ethics on Human Experimentation (Krugman, Giles and Hammond[53,54]).

The studies at Willowbrook confirmed that there are two distinct epidemiological, clinical and immunological types of infective hepatitis. One type of illness, induced by MS-1 serum, resembled closely classical hepatitis A. It was characterized by a short period of incubation and it was highly infectious through natural contact. The results of biochemical tests were also characteristic of infectious hepatitis. The second type of infection, induced by MS-2 serum, resembled hepatitis B. This infection had a long incubation period, and biochemical tests of liver function were, on the whole, more typical of the findings in serum hepatitis. It was also observed, contrary to the general view, that the serum hepatitis-like infection was transmissible via the oral route, although it was less infectious even for close contacts. Other experiments showed that the children inoculated with the infectious hepatitis type MS-1 serum had homologous immunity, but there was no cross-immunity between MS-1 infection and the infection caused by MS-2. This apparent lack of heterologous immunity between the two types of agent confirms the volunteer experiments conducted during the 1940's and early 1950's in Great Britain and in the United States. Krugman and his associates suggested that the existence of these two distinct varieties of infection was responsible for the occurrence of second attacks of jaundice at Willowbrook, often within one year of the first illness, in 5.5 percent of the 1153 patients who suffered from icteric hepatitis.

The studies in experimentally infected volunteers also revealed the presence of viremia during the long incubation period of the illness, namely 87 days before the onset of hepatitis (Neefe, Stokes, Reinhold and Lukens[55]) 60 and 16 days before the appearance of jaundice (Paul, Havens, Sabin and Philip;[56] Havens[50]). Evidence that a prolonged carrier state for as long as five years may result in some patients, with or without signs of liver disease, was provided by Stokes and his associates,[57] by Neefe, Norris, Reinhold, Mitchell and Howell,[58] and by Murray, Diefenbach, Ratner, Leone and Oliphant.[59] Zuckerman and Taylor[60] demonstrated in 1969 persistent carriage of hepatitis B antigen for over 20 years. Murray *et al.*[61] also recorded the case history of a donor in whose blood hepatitis

B virus was found 135 days after he donated blood which was subsequently incriminated as the cause of hepatitis in the recipient. The same donor later developed hepatitis himself. Viremia was demonstrated once again, on this occasion six months after recovery from the illness. These observations confirmed long held epidemiological and clinical impressions that a prolonged carrier state of the virus may persist in the blood of at least some patients. With the development of sensitive laboratory techniques for the detection of antigens and antibodies associated with hepatitis B virus it is conservatively estimated that there are some 112 million carriers of hepatitis B surface antigen in the world today.

As far as hepatitis A is concerned the data are incomplete. Early volunteer experiments revealed that viremia and the excretion of infective virus in the feces may occur in most patients during three to four weeks of the incubation period and during the acute phase of infectious hepatitis. However, the excretion of virus may be prolonged. Thus, Capps, Bennett and Stokes[62] demonstrated the presence of virus in the feces of one infant with chronic hepatitis five months after onset of the disease and in another with chronic hepatitis 15 months after acquiring the infection. Murray *et al.*[61] also reported viremia in a patient eight months after complete recovery from infectious hepatitis. It was suggested that a prolonged carrier state can therefore occur in hepatitis A, but there is considerable epidemiological evidence that a chronic carrier state is much more frequently associated with hepatitis B. It is not inconceivable that the carrier state previously ascribed to hepatitis A might have been associated with hepatitis B.[63]

The era of tissue culture

With the development of sensitive serological techniques, a number of viruses were isolated from patients suffering from hepatitis, resulting in a heterogeneous collection of viruses which have been referred to as the "hepatitis candidate viruses." Some of these have been identified and classified; others have not. Some have been identified as other micro-organisms including a strain of Mycoplasma and an amoeba of the *Hartmanella* genus. Several studies are summarised below; an extensive review was published in 1970 by Zuckerman.[64]

Essen and Lembke[65,66] claimed that hepatitis virus could be cultured in embryonated hens' eggs and that the contents of infected eggs induced hepatitis in volunteers. Henle *et. al.*[67] also reported in 1950 the passage of human hepatitis viruses in tissue culture and later the successful cultivation of the hepatitis virus in embryonated eggs. Subsequent studies, however, using eggs were unrewarding.

Undoubtedly the most widely known hepatitis candidate viruses were those described in 1956 by Rightsel and his colleagues.[68] A number of filterable agents were isolated from the sera and feces of patients with hepatitis using the Detroit-6 epitheloid cell line which was originally derived from human bone marrow. Technical difficulties were soon encountered, not least of which was the tendency of the cells to undergo spontaneous degeneration. Futhermore, human serum was required for growth of the cells, which would tend to invalidate any experimental work with the isolated agents, and, in particular, transmission experiments in human volunteers. Later the cells were cloned and the human serum was replaced by unfiltered fetal calf serum. In 1961, Rightsel *et al.*[69] published details of the tissue culture studies, the virus isolation and the characteristics of the viruses. Limited

clinical trials in human volunteers were also undertaken, and at least three serotypes of virus could be identified (Boggs *et al.*[70]). Two of the strains (AR-17 tissue culture passage virus and WW-55 plasma) induced hepatitis with jaundice in some volunteers. Difficulties in reproducing these tissue culture results were experienced in other laboratories.[64] In 1965, Cole[71] reported the isolation of viral agents in Detroit-6 cells from the serum of 27 out of 28 patients with hepatitis. These results could not be confirmed by Cross and Marmion.[72] They were unable to find a correlation between the cytopathic effect produced in Detroit-6 cells and the disease of the patient, and it was further noted that even the same specimens when tested under different code numbers gave different results.

Hillis[73,74] obtained epidemiological evidence that chimpanzees may act as temporary carriers of the human hepatitis virus. It therefore seemed possible that the hepatitis might replicate in tissue culture cells derived from chimpanzee organs. Prelimiary studies indicated that two agents, derived from 2 to 12 sera obtained from patients during the acute phase of hepatitis, destroyed primary chimpanzee kidney cells. Further isolation of virus was reported in 1963 by Hillis[74] from the feces of chimpanzees implicated in the transmission of hepatitis to man, but the serum neutralization tests have been disappointing, and the use of primary chimpanzee kidney cultures for the isolation of human hepatitis virus has not proved successful.

In 1961, Davis[75] described the isolation of cytopathic agents from 14 to 22 young Indian children involved in an outbreak of hepatitis A at the San Carlos Reservation in eastern Arizona during the autumn of 1959. These cytopathic agents were isolated from feces using an epithelial cell line derived from human embryo lung. Similar agents were isolated from another epidemic on another Indian reservation two years later. Attempts to identify these agents as enteroviruses were unsuccessful and they were named the San Carlos viruses. Fifteen strains were isolated from 45 fecal specimens. Subsequently these strains were identified as adenoviruses types 1, 2, and 3 (Hatch and Siem[76]). Zuckerman *et al.*[77] infected tissue cultures of human embryo hepatocytes with the San Carlos viruses. Examination by thin section electron-microscopy of hepatocytes infected with San Carlos virus 6 revealed the presence of two virion types of markedly different size (Zuckerman *et al.*[78]). The larger particle size was 65 to 70nm, and smaller virus-like particle measured 30 to 40nm. The nature of the second virion type is not yet clear, but its size is certainly much larger than the diameter of 22 to 24nm which has been found with the known adeno-associated viruses.

Zuckerman[79] reviewed the techniques which have recently been developed for cultivation of human and nonhuman primate liver cells in tissue and organ culture. Progressive noncytocidal involvement of the normal cytoplasmic and nuclear components of cultured liver cells has been demonstrated by specific attachment of fluorescent antibody to hepatitis B core and surface antigens after inoculation of the cultures of human origin with known infective sera and with clinical material. Hepatitis B surface antigen may also be produced, although infrequently, in inoculated liver organ cultures, but serial passage has not been achieved. Serial passage of hepatitis B virus has been reported with fragments of human embryo liver cultivated on the chorioallantoic membrane of the developing chick embryo. In other experiments, virus-like particles have been localized in hepatocytes of cultured explants of liver biopsies obtained from infants with chronic hepatitis B

antigenemia. It is clear, however, that further studies are required to determine whether cultivation of hepatitis B virus can be firmly established in readily available cell and organ cultures. No progress has been reported on the cultivation of hepatitis A virus.

Serological tests for viral hepatitis

Havens[80] reviewed the early attempts by J.S.H. Gear to devise a specific serological test for the diagnosis of hepatitis B. A precipitin and complement-fixing antibody was found in the convalescent serum of some patients with hepatitis following immunization with yellow fever vaccine. Gear[81,82] also referred to this precipitin reaction demonstrated in some soldiers who contacted hepatitis following immunization against yellow fever in 1942. This antibody reacted with an antigen found in the acute phase serum. Similarly, precipitating and complement-fixing antibodies were demonstrated in convalescent phase sera which reacted with antigen(s) in acute phase sera and in saline extracts of normal human liver and liver from patients with hepatitis (Sawyer *et al.*,[83] and Eaton, Murphy and Hanford,[84]). Pollard and Bussell,[85] in 1953, also described a substance in the acute phase serum of a patient with hepatitis B which fixed complement with sera from patients convalescent from hepatitis B, but not with sera from patients recovering from hepatitis A or with other forms of jaundice. However, it was not until the discovery of Australia antigen (hepatitis B surface antigen) that a specific and reproducible serological test finally became available for the diagnosis of hepatitis type B.

Discovery of Australia antigen

Polymorphism is defined as the occurrence in the same habitat of two or more inherited forms of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation. In polymorphic traits, two or more of the genotypes determining variation of the trait are common to the population. Polymorphisms are believed to arise as a result of selective differences between genotypes, and they provide convenient systems for the study if inherited discontinous biochemical variation in man. Included in such systems are the red blood cell antigens (ABO, MNS, P, Rhesus etc.), sickle cell hemoglobin, haptoglobin, transferrin, glucose-6-phosphate dehydrogenase deficiency, gamma globulin groups, and so on. Allison and Blumberg[86] argued on this basis that patients who are transfused would be likely to receive blood containing proteins which they had not inherited or acquired, since donor blood is commonly typed only for the major red blood cell antigens. Some of these differences might, therefore, be antigenic and lead to the development of antibodies in the transfused patients. A systematic investigation of the serum of transfused patients was begun, using the simple two dimensional micro-Ouchterlony immunodiffusion technique.

After the examination of 13 sera transfused patients in the centre well and a panel of sera from different geographical areas in the peripheral wells, serum from one of the transfused patients was found to contain a precipitin which reacted with some of the sera in the panel. It was soon demonstrated that this antiserum defined a system of inherited antigenic specificities. A search for additional such systems was initiated, and in 1963 two sera from multiply transfused American hemophiliacs were found to give a single precipitin line with only 1 of 24 sera in the test panel. The antigen in this single serum contained little or no lipid and clearly differed from the serum β-lipoproteins. Since the reacting serum was obtained from an

Australian aborigine, the antigen was named "Australia" antigen (Blumberg, Alter and Visnich[87]). Subsequent studies on the distribution of the antigen in normal population in different geographical areas of the world revealed that this antigen was very rare, or absent, in normal North American and European communities, but that it occurred frequently in the serum of apparently healthy people living in the tropics and Southeast Asia (6 to 25 percent). The antigen was found frequently in the serum of patients with acute leukemia. It was suggested in 1965 that the presence of Australia antigen may be of value in the early diagnosis of leukemia, and, further, that the antigen may be related to the virus postulated as the cause of leukemia (Blumberg[88]). Evidence was also presented that Australia antigen was inherited as a simple autosomal recessive trait. A corollary of this hypothesis was that individuals at high risk of developing leukemia have an increased frequency of persistent Australia antigen. Patients with Down's syndrome are known to have a high risk of leukemia, and up to 30 percent of sera from such patients were found to contain Australia antigen (Blumberg *et al.*,[89] Sutnick *et al.*[90]).

In 1966 one of Blumberg's patients with Down's syndrome, who on initial examination did not carry the Australia antigen but in whose serum the antigen was subsequently found, was investigated further. Because many serum proteins are manufactured in the liver, liver function tests were carried out. These investigations and a liver biopsy revealed that this patient developed hepatitis concomitant with the appearance of Australia antigen in his blood. In the same year a technician working with Australia antigen in Blumberg's laboratory developed loss of appetite and dark urine. Australia antigen was detected in her blood for only a single day and she subsequently developed a mild hepatitis (Blumberg[88]). Sera of patients with viral hepatitis were examined for Australia antigen,and the antigen was found in 10.4 percent. Subsequent studies revealed a close association between Australia antigen and hepatitis B, and a new era in hepatitis research began.

The decade of nonhuman primates

Hepatitis A: Hillis[73,74] reported an epidemic of hepatitis A among handlers of young, newly imported chimpanzees at the U.S. Air Force Missile Development Center. Since then, over 40 outbreaks involving over 170 human cases have been reported to the Communicable Disease Center, Atlanta. The illness in man is usually mild and is indistinguishable from hepatitis A. The incubation periods have generally been 3 to 5 weeks; secondary spread does not seem to occur. The highest attack rates are in persons having direct contact with newly imported animals. Persons in contact with the animals two months after importation have not developed hepatitis. Most human cases have been associated with chimpanzees, although woolly monkeys, gorillas, gibbons and Celebes apes have been implicated. The monkeys do not suffer from a recognizable clinical illness. Contact with man before importation and contact with other primates during and after importation may result in infection. It has been suggested that the human hepatitis virus is merely a passenger in the gastrointestinal tract of nonhuman primates, although a subclinical infection is the more likely.

Observation of non-human, primate-associated hepatitis restimulated attempts to transmit human viral hepatitis to different species of monkeys (reviewed in 1970 by Deinhardt[91]). Working on the assump-

tion that primates which had little or no contact with man were unlikely to have acquired immunity to the human hepatitis virus through subclinical infection. F. Deinhardt and his associates began to experiment with marmosets, a species of small South American monkeys[92]. These animals have little contact with man, and serological surveys of antibody confirmed that naturally acquired infection to common human viruses was rare. Biochemical and histological changes compatible with hepatitis were found in two species of marmosets, *Sanguinus nigricollis* and *S. fuscicollis,* after the inoculation of acute phase serum or plasma from patients with viral hepatitis. There was no clinical evidence of infection in the marmosets, but liver damage was produced regularly in series five times, from marmoset to marmoset, using a pool of animal serum.

In studies using different species of marmosets, susceptibility to infection was demonstrated in almost all the animals, and this implied that spontaneous hepatitis was not frequent, especially since resistance to re-infection on challenge was also shown. The histological lesions in the liver of the cotton-topped tamarin marmosets were not the same as those found in inoculated marmosets of three other species. Holmes *et al.*[93] found that marmosets inoculated with well-documented acute phase hepatitis A plasma from three human volunteers developed hepatitis, whereas other marmosets injected with pre-infection plasma from the same volunteers showed no evidence of liver damage.

In 1970 Lorenz *et al.*[94] provided additional evidence that marmosets of the species *Saguinus mystax* can serve as animal models to study human infectious hepatitis. Biochemical evidence of liver damage and hepatic lesions were induced by the injection of serum from a pool of sera from volunteers with experimentally transmitted hepatitis A and by serum from pooled marmoset sera which had been passed five times in marmosets. Two marmosets which were previously inoculated with infectious marmoset serum did not develop evidence of hepatitis when challenged with human hepatitis A serum. Holmes *et al.*[95] published the results of four separate experiments on the induction of hepatitis in marmosets of the species *S. fucicollis* or *nigricollis* or *oedipus oedipomidas,* using human hepatitis A serum or plasma specimens under code. Infective material induced hepatocellular damage in 33 out of 43 inoculated animals, whereas normal serum or plasma did not cause liver damage in any of the 42 control marmosets.

More recently, Mascoli *et al.*[96] reported the results of their experiments conducted over a period of 5 years with 274 marmosets (mainly of the species *Saguinus mystax* and a few *S. nigricollis).* The marmosets were inoculated intravenously with blood from human cases of hepatitis A or hepatitis B infection. Goat serum, human immune globulin or saline solutions were used for control. It was found that the indispensable element for the induction of hepatitis in marmosets, as judged by elevation of serum glutamic pyruvic transaminase and serum isocitric dehydrogenase and histological changes in the liver, was the introduction of blood from patients with hepatitis A, but not from hepatitis B infections nor the control materials. For special control, serial passage was carried out in marmosets of a pool of sera obtained prior to inoculation from marmosets in which the hepatitis A agent was subsequently propagated. All marmosets in all passages retained normal serum isocitric dehydrogenase levels, except for one animal in the third passage. This enzyme elevation was probably nonspecific, since the animal died shortly after this episode, and histological evidence

of hepatitis was not found. Blood specimens from five out of seven patients with acute hepatitis A caused hepatitis in marmosets. Blood samples taken from patients 22 to 29 days before the onset of illness gave negative findings in 70 marmosets. Pools containing samples from patients during the acute illness, or 29 to 113 days after onset, yielded positive serum enzyme levels in 24 of 119 animals. It is noted that the liver histology and serum enzyme elevations were not always in total agreement, as shown by the appearance of lesions in 4 out of 112 marmosets in which there were no significant enzyme changes. The hepatitis A agent was passaged successfully in marmosets four times. It was concluded that the marmoset is highly reliable for detecting, propagating and studying human hepatitis A. Spontaneous hepatitis was not encountered in marmosets.

Provost *et al.*[97] used the CR 326 strain of hepatitis A virus of the fourth passage in marmosets for determining the physical and chemical properities of the agent. Filtration data showed the particle size to be in the range of >25nm <50nm. The virus was stable to ether and stable in acid (pH 3.0 for three hours at room temperature) and resisted heat for one hour at 60°C in sealed glass containers. The results were evaluated by intravenous inoculation of 1.0ml of the treated preparations to each of 12 marmosets per sample. Susceptibility of two species of marmosets to hepatitis A was also evaluated. It was found that 80 percent of *Saguinus mystax* developed hepatitis by the eighth week after inoculation compared with only 33 percent of *Saguinus nigricollis*. A pool of sera of the fourth passage of strain CR 326 in *S. mystax* marmosets was used for serum neutralization tests. The tests were carried out with eight paired sera from patients with hepatitis A and two patients with hepatitis B. These included paired sera under code from three human volunteers inoculated with the MS-1 strain of hepatitis A and one inoculated with the MS-2 strain of hepatitis B. In addition, three samples of pooled human immunoglobulin were used for neutralization studies. It was found that all patients with classical hepatitis A infection developed antibody which neutralized the CR 326 marmoset strain; but there was no such antibody response in patients with hepatitis B infection. All three samples of human immunoglobulin neutralized CR 326. Neutralization was highly effective, since marmosets inoculated with the neutralized virus (serum-antiserum mixture) remained susceptible to reinfection by CR 326. In contrast, marmosets given non-neutralized virus mixture were immune to reinfection. The precise limits of sensitivity and reliability of this serum neutralization test are as yet undetermined, in view of the large numbers of assays which are required.

In 1973, Holmes *et al.*[98] reported the results of attempts to neutralize hepatitis A virus infection in marmosets with convalescent human serum. Acute phase serum or plasma from human volunteers inoculated with the MS-1 strain of human hepatitis A, or the infectious fractions of such serum or plasma prepared by density gradient separation in caesium chloride, were mixed in a 1:1 ratio with pre-inoculation or convalescent serum from one of the volunteers. The mixtures were incubated for 16 to 18 hours at 4°C, and then 0.5ml inoculated intravenously, under code, into groups of marmosets *(S. nigricollis* and *S. fuscicollis).* The convalescent serum neutralized almost completely the infectivity of the acute phase serum or the infectious fractions prepared from it, whereas infectivity was unaffected by incubation with human albumin or by incubation with the pre-inoculation serum.

The availability of the marmoset model is of practical importance in investigating the pathogenesis of hepatitis A, for detecting and identifying the virus, as well as for determining its biophysical and biochemical properities (Deinhardt *et al.*[99]), and eventually for the evaluation of potential vaccines.

Hepatitis B

There have been many attempts to transmit hepatitis B virus to nonhuman primates, and these investigations have yielded, until recently, equivocal or negative results. The findings of hepatitis B antigen and antibody in the serum of a small proportion of chimpanzees, orangutans and gibbons renewed interest in the possibility that such nonhuman primates might serve as a suitable experimental model for hepatitis B. Recent studies, employing sensitive assay methods for hepatitis B antigen and antibody, have established the susceptibility of the chimpanzee in particular to infection with the human hepatitis B virus. It would seem that much of the difficulty which had been experienced in the past was due to the unknown susceptibility of the animals before experimentation.

Hepatitis B antigen and hepatitis B antibody have been detected in 6 to 12 percent of captive chimpanzees when tested by relatively insensitive techniques. Most of the animals appear to be healthy carriers of the antigen. Hepatitis B antibody has been detected in a significant proportion of captive nonhuman primates when sensitive techniques such as passive hemagglutination and radioimmunoassay had been used. Antibody was found in the chimpanzee, orangutan, gibbon, baboon, Celebes ape, patas monkey, vervet, several species of macaque, mangabey, and langur and in a number of species of New World monkey. Antibody was found in approximately 50 percent of chimpanzees examined but in less than 10 percent of most Old World and New World monkeys. The frequeny of hepatitis B antibody in both human and chimpanzee populations seems to increase with age. The prevalence of hepatitis B antigen and antibody in chimpanzees in the wild is not known. The subject has recently been reviewed by Zuckerman.[100]

The high frequency of naturally acquired antibody to hepatitis B virus among the apes, and the relatively mild nature of the infection in nonhuman primates were most probably responsible for the apparent failure of previous attempts to transmit this infection to these animals. Maynard, Berquist, Drushak and Purcell[101] inoculated two chimpanzees, inferred to be susceptible to hepatitis B because of the absence of this antibody by highly sensitive techniques, with 1ml of a 1:10 dilution of human plasma containing hepatitis B antigen and known to have caused hepatitis in man. Hepatitis B antigen was detected in the serum of one chimpanzee 70 days after inoculation, and antibody was detected 36 days later. The antibody was first detected by passive hemagglutination, with the maximum titre occurring 226 days after inoculation. Antibody detected by radioimmunoassay did not develop until seven days after the appearance of the hemagglutinating antibody (day 113) and precipitating antibody was not detected until 151 days after inoculation. Antigen was not detected in the second chimpanzee, but hemagglutinating antibody developed 27 days after inoculation, radioimmnoassay antibody on day 32 and precipitating antibody after 151 days. Neither chimpanzee developed clinical, biochemical or histological evidence of hepatitis.

Barker *et al.*[102] extended these studies. Six chimpanzees were selected for transmission studies of hepatitis B infection because they were seronegative for hepatitis B antigen and antibody using sensitive techniques. In the first experiment two chimpanzees were inoculated subcutaneously with an NIH plasma pool, which was shown many years ago to be highly infectious for man. In other experiments chimpanzees received plasma containing hepatitis B antigen collected 21 weeks after inoculation, from one of the animals, and other animals were given material containing antigen from fractions obtained by rate zonal centrifugation in sucrose and from an isopycnic banding in a sucrose gradient. Five of the six chimpanzees developed evidence of hepatitis B infection. Hepatitis B antigen was detected, by immunofluorescence, in association with almost all hepatocytes of a chimpanzee which was a chronic carrier of the antigen. Distribution of the antigen was most prominent in the region of the sinusoidal aspect of hepatocyte surfaces of the subendothelial space. Hepatocyte surface localization was also observed within the lobular plates. The antigen was also seen, although less frequently, within the cytoplasm of hepatocytes. Weak positive immunofluorescent reactions for the core antigen were seen very rarely in the nuclei of hepatocytes. The reaction was not neutralized by purifed 22nm particles of the antigen. In a biopsy from another chimpanzee, the core antigen was demonstrated by immunofluorescence in approximately 1 to 4 percent of hepatocytes. This reaction could not be neutralized by blocking experiments with human or goat hepatitis B antibody, but neutralization was demonstrated by blocking with hepatitis B core antibody. Thus, the core antigen and outer coat hepatitis B antigen appeared to be sterically distinct and anatomically segregated. Examination by electron microscopy of liver biopsy from the latter chimpanzee, 27 and 28 weeks after inoculation, revealed groups of 22 to 28nm virus-like particles in the nuclei of some apparently normal hepatocytes.These particles resembled those described in the cytoplasm and nuclei from human cases of hepatitis B. Very rarely a particle was observed that measured approximately 35nm in diameter containing a 22 to 28nm core surrounded by an outer coat. Of the three remaining chimpanzees, two developed transient circulating hepatitis B antigen followed by hepatitis B antibody, and the third developed the antibody approximately three months after inoculation, without any biochemical or histological evidence of hepatitis. It was pointed out that so far the chimpanzee is the only animal to develop a disease resembling hepatitis B, manifested by elevated levels of serum enzymes and liver damage after experimental infection with hepatitis B virus; thus the chimpanzee would appear to be a promising model for investigating protective immunity as well as for the study of the pathogenesis of the carrier state.

It is expected that progress in the use of nonhuman primates for the study of human hepatitis will be impeded by a shortage of suitable susceptible animals, particularly the apes. Such studies should not be undertaken therefore without recognizing the problems involved in acquiring and maintaining susceptible animals and in documenting the often very mild and evanescent infection.

The golden era of the electron microsocpe

Viruses were among the first objects to be examined in the electron microscope over 30 years ago, yet little information was obtained on the fine structure of viruses,

except in the bacteriophage field, until the introduction in 1959 of the negative contrast technique for high resolution microscopy. This method utilizes the principle of surrounding within a rigid electron-dense material particles such as viruses. Usually there is very good preservation of the biological material under test with minimum distortion of structure. The technique of immune electron microscopy, a procedure which concentrates particles within an immune complex, provides an additional degree of sensitivity. The techniques of preparing serum specimens for examination of hepatitis B antigen by negative staining have been described in detail elsewhere (Zuckerman[103]).

Examination of hepatitis B antigen in the electron microscope, after negative staining, revealed a remarkable morphological heterogeneity of particles consisting of three principal virus-like structures (Fig. 1). The main antigenic constituent is a spherical pleomorphic particle 16 to 25nm in diameter. The presence of tubular forms averaging 22nm in diameter and often several hundred nanometers in length is a characteristic feature. The tubular forms usually display a regular transverse periodicity of 3nm, and bulbous swellings are frequently seen at either or both ends of the tubules. The third type of particle, the Dane particle, is also spheroidal, measuring about 42nm in diameter, with a 28nm core, a 2nm shell and an outer coat 7nm in thickness.

Detergent treatment of pellets of hepatitis B antigen obtained by ultracentrifugation of whole serum containing the large 42nm particles resulted in separation into an outer coat of antigen and an inner component or core which was about 27nm in diameter (Almeida *et al.*[104]). Immune electron microscopy revealed that antibody present in the serum of patients after recovery from hepatitis B reacted with the core, but not with

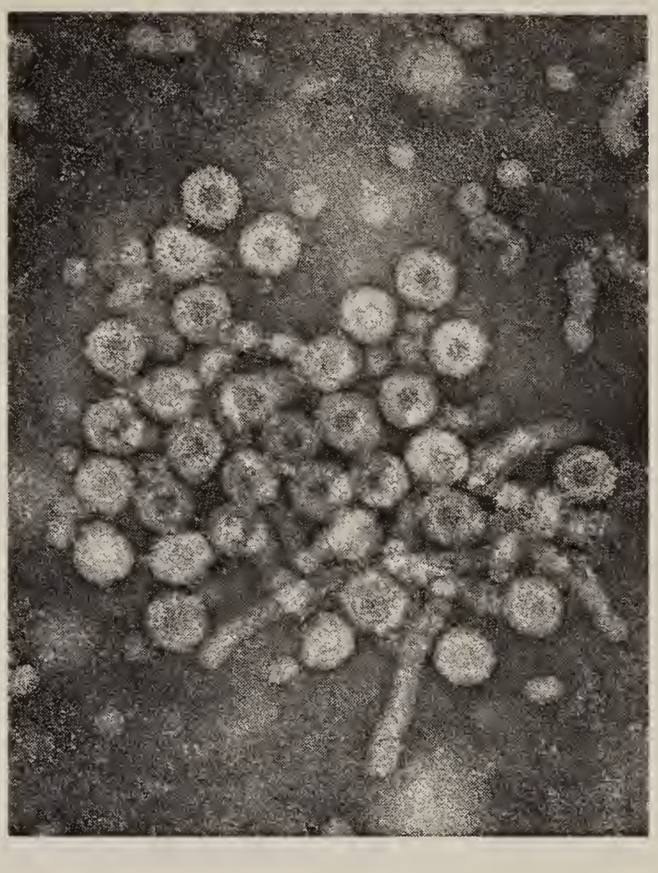


Fig. 1—The morphology of hepatitis B antigens showing three distinct entities, small 22nm spherical particles, tubular forms of varying length and a large 42nm double-shelled spherical particle. Original magnification x 252,000 (Reproduced with permission from *Human Viral Hepatitis,* North Holland and American Elsevier, 1975).

the surface hepatitis B antigen component, to yield immune aggregates resembling those seen in homogenates of liver taken *post mortem* from patients with type B hepatitis. An important observation was that antibody to the core was absent from the prehepatitis sera from the same convalescent patients. Demonstration by immune electron microscopy that the core antigen and its antibody are immunologically distinct from hepatitis B surface antigen and the surface antibody has been confirmed by immunofluorescent studies localizing the core antigen in liver tissue, by complement fixation, by counterimmunoelectrophoresis, and by radioimmunoassay.

It is of considerable interest that particles which were virtually identical to hepatitis B antigen and consisting of

three morphological entities: small spherical particles, rod-like structures, and double-shelled spheroidal (later named "Dane") particles were first described in 1966 by Harris *et al.*,[105] using electron microscopy, after differential centrifugation of plasma obtained from two patients — one with chronic lymphocytic leukemia and the other with lymphocytic lymphosarcoma. These particles were recognized then as unique virus-like structures, and in one patient the particles persisted over a period of months.

The morphological complexity of the particles associated with hepatitis B is matched by the complex antigenic reactivities of the coat protein which is associated with the small spherical particles, the tubular forms and the surface of the Dane particles (Fig. 2.). All these structures share a common group specific antigen *a* and the particles generally carry at least two subdeterminants, either *d* or *y*, which usually behave in a mutually exclusive manner although carried on the

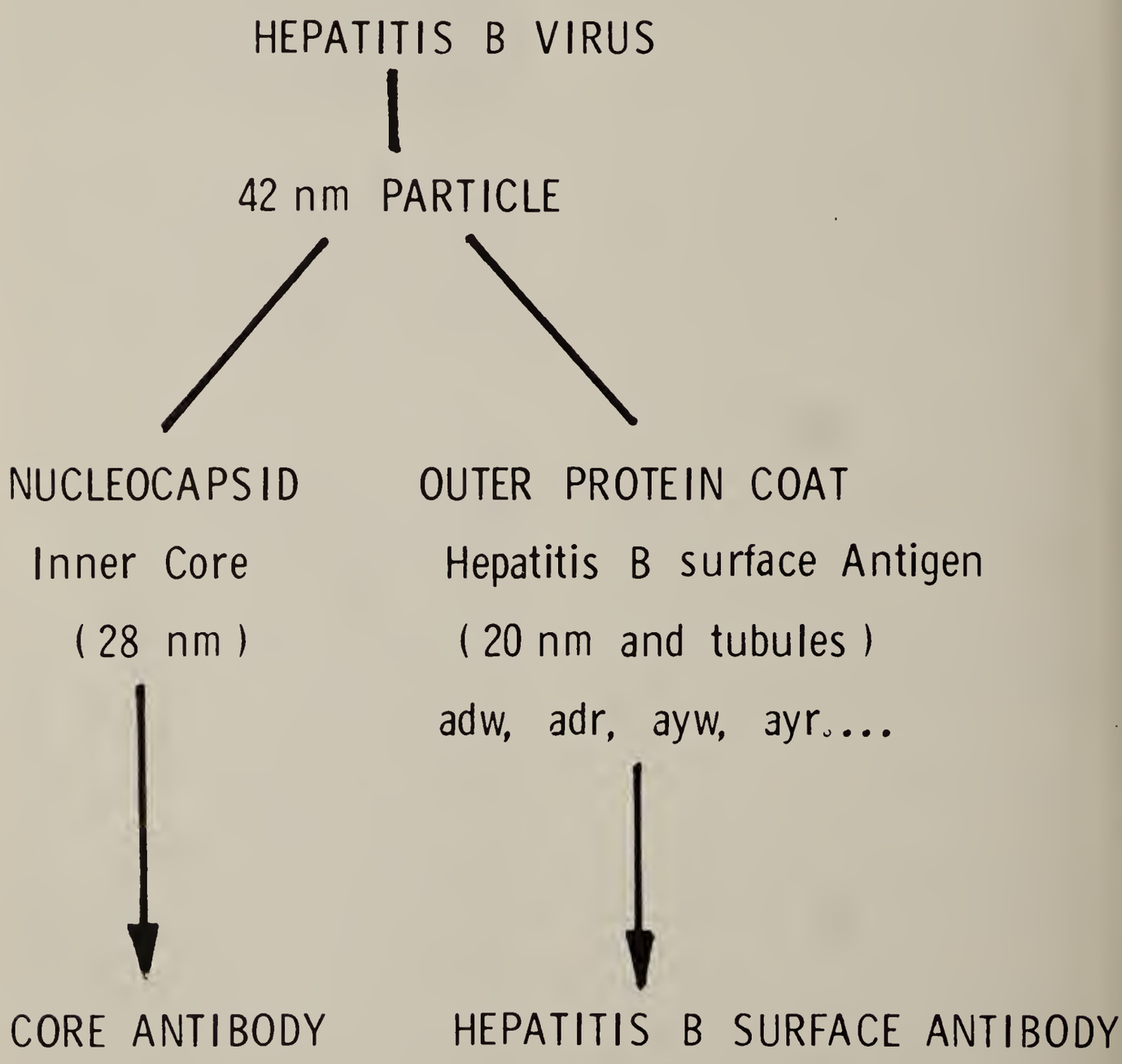


Fig. 2—A schematic presentation of the complexity of hepatitis B antigens.

same antigen particle, and either *w* or *r*. There is evidence that the subtypes are the phenotypic expressions of distinct genotype variants of hepatitis B virus. Four principal phenotypes are currently recognized: *adw, adr, ayw* and *ayr*, but others are not precluded. Indeed, complex permutations of these subdeterminants and new variants have been described, all apparently on the surface of the same physical particles. A remarkable geographical pattern of distribution of hepatitis B subtypes has emerged with four global zones, where there is an excess of one subtype and regions where a mixture of subtypes is common. These subtypes provide valuable epidemiological markers and offer a method for distinguishing several sources of infection. These surface antigenic reactivities do not appear to be associated with particular clinical forms of liver disease.

Another antigen-antibody system which is associated with molecules distinct from particles of hepatitis B antigen has also been described.[106] This precipitating antigen, which was termed *e*, was found in sera containing hepatitis B surface antigen. The *e* antigen differed markedly from the previously described determinants of the surface antigen. Paradoxically, antibody against *e* is found in sera from healthly carriers of the surface antigen. The *e* antigen appears to be intimately associated somehow with the pathogenesis of liver damage. Recently, the *e* antigen was found to be significantly more common in patients with chronic hepatitis and cirrhosis with persistent hepatitis B antigenemia than in patients with acute viral hepatitis. Furthermore, the *e* antigen seems to be a valuable and important prognostic marker, since progression to chronic liver disease has been recorded by serial liver biopsies in consecutive patients with surface antigen-positive acute hepatitis associated with the *e* antigen. The clinical significance of the *e* antigen is supported by differences in the clinical, biochemical and histological findings between patients with the *e* antigen and those without during the initial phase of viral hepatitis. The exact nature of the *e* antigen is uncertain; it might be a host antigen produced by virus-infected liver cells or it might be related to another antigenic constituent of the infecting virus, perhaps the core of the 42nm Dane particle.

The electron microscope has also been employed successfully in identifying hepatitis A virus particles. Feinstone *et al.*[107] examined by immune electron microscopy extracts of feces obtained before infection or during the acute illness from adult volunteers who were infected orally or parenterally with the MS1strain of hepatitis A virus. Virus-like particles measuring 27nm in diameter were found in a number of fecal specimens during the acute phase of illness. Development of antibodies to these antigen particles has been demonstrated. These findings have been confirmed in other laboratories. Almeida *et al.*[108] found that the same fecal material used by Feinstone *et al.* contained several small cubic virus-like particles which varied slightly but significantly in diameter size. Particles of 22nm, 27nm and 30nm in diameter were distinguished. Thorton *et al.* [109] collected feces and serum over a period of eight weeks after inoculation of a susceptible chimpanzee with feces obtained from an adult human volunteer infected in the United States with the MS-1 strain of hepatitis A virus. Serum enzyme levels became abnormal after three weeks and remained elevated until the sixth week. Examination of fecal extracts by immune electron microscopy revealed the presence of a small cubic virus that could be detected as early as nine days after infection and persisted until the 28th day. The particles were distinctive and easy to identify (Fig. 3), but even within single aggregates, particle diameters ranged from 24 to 29nm.

The availability of hepatitis A antigen has permitted the development of specific serological tests for hepatitis A antibodies. Techniques include immune electron microsopy, complement fixation, immune adherence hemagglutination and radioimmunoassay. Enzyme-linked immunoadsorbent assays are under investigation.

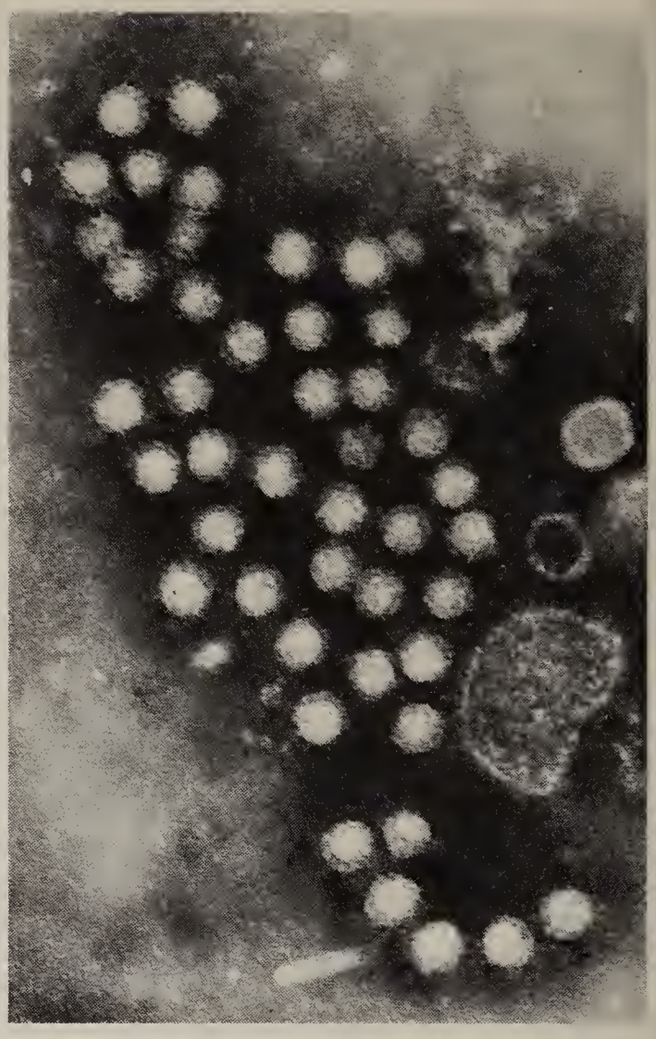


Fig. 3—Virus-like particles measuring 27nm in diameter found in fecal extract from a chimpanzee infected with human hepatitis A. Original magnification x 275,000 (Electron micrograph from a series by A. Thornton, A. J. Zuckerman and J. D. Almeida).

Vaccine studies

Viral hepatitis type B is worldwide in distribution and there is a particular need for a vaccine for those groups at increased risk of aquiring this infection. These groups include health care personnel, children of mothers who become infected during pregnancy and of mothers who are carriers, patients residing in large institutions and the personnel providing for their care, patients on maintenance hemodialysis and those requiring repeated blood transfusions or the administration of blood products, and persons living in certain tropical areas where sanitation is poor.

The repeated failure to passage hepatitis B virus serially in tissue or organ cultures (Zuckerman[79]) has hampered progress towards the development of a safe and effective vaccine. Attention has therefore been directed recently towards the use of other preparations for active immunization against hepatitis B (Zuckerman and Howard[110,111]).

Active immunization was attempted by Krugman, Giles and Hammond[53,54] using as the immunogen a known infective human serum (MS-2 serum) that contains hepatitis B virus. The serum was diluted 1 in 10 in distilled water and heated at 98°C for one minute. Serum treated in this manner was not infective and it successfully prevented or modified hepaitis B in 69 percent of susceptible persons inoculated with the heated serum and challenged with the original infective serum four to eight months later. Results with the heat-inactivated serum were essentially the same after one, two or three inoculations. In other studies carried out by Soulier *et al.*[112] serum containing hepatitis B surface antigen obtained from a healthy carrier was heated at 60°C for 10 hours, but the infective

agent was not completely inactivated as shown by the acquisition of antigen and elevated serum transaminase levels in a proportion of the recipients. Furthermore, this is a crude way of inducing immunity, and it is unlikely to be accepted for general use. Nevertheless, the work of Krugman and his colleagues laid the foundations for unconventional hepatitis B immunogens that could be used as vaccines.

Isolated viral coat protein challenges the immune mechanism of the body in the same way as the intact infectious agent, and the possibility of using purified spherical 20nm hepatitis B surface antigen particles, free of detectable nucleic acid, seems attractive. Such experimental vaccines have been prepared, and a limited number of susceptible chimpanzees were shown to be protected by such immunogens.[113] These studies, although encouraging, were by no means comprehensive, and some doubts linger regarding the possible harmful induction of immunological reactions to host proteins which may be closely associated with or incorporated in the 20nm spherical particles of hepatitis B surface antigen. These host proteins may include various pre-existing structures of the liver cell. Neurath *et al.*[114] recently reported that antigenic determinants related to human plasma proteins are constituents of hepatitis B surface antigen. These determinants appear to be related to antigenic specificities or prealbumin, albumin, apolipoproteins C and D, and the gamma chain of immunoglobulin G. Other studies indicated that the larger polypeptides of purified surface antigen are probably adsorbed serum proteins which are necessary for the preservation of antigenic activity. Finally, the close association of the surface antigen with normal serum components has been an acknowledged difficulty in developing purification techniques for separation of the antigen.

Demonstrable and significant levels of carbohydrate in purified fractions of the surface antigen have also been found. The total carbohydrate content of purified small antigen particles is reported as 3.6 to 6.5 percent. Three glycoproteins were also found as well as a glycolipid and three major phospholipids (Chairez *et al.*[115] and Steiner *et al.* [116]). The carbohydrate may be necessary for maintaining the structure and functional integrity of the antigenic determinants, or the carbohydrate itself may constitute a major antigenic determinant. The carbohydrate might have a novel haptenic specificity which is either virus-coded or virus-induced host cell-coded. Alternatively, the carbohydrate, and some lipoprotein components, might simply be derived from the host cell membranes as the mature virus particles are released. There may be some similarity between such a carbohydrate hapten, or the lipoprotein components, and those carbohydrate and lipoprotein antigens of normal cell surfaces, leading to a degree of tolerance because of a close antigenic resemblance between hepatitis B surface antigen and "self" antigens.

Alternatively, an autoimmune reaction may be initiated. Such an autoimmune reaction may be induced by hepatitis B infection because of a change in antigenicity of the hepatocyte cell membranes, due to alteration of existing antigens or the appearance of viral determinants. T lymphocytes responsive to such new antigenic determinants could promote a B cell response to unaltered "self" antigens. The synthesis and release of the resulting auto-antibody is subject in turn to control by suppressor T cells. These complex interactions between T and B cells, could be of fundamental importance in the pathogenesis of chronic liver disease.

The development of cell-mediated immunity to hepatitis B virus antigens during the acute stage of the disease, its persistence during convalescence and disappearance after recovery, and its absence in persistent asymptomatic carriage of the surface antigen suggest that cell-mediated immunity may be involved in terminating viral infection and, under certain circumstances, in promoting hepatocellular damage.

Liver-specific lipoprotein is a macrolipoprotein which is thought to be a normal constituent of the hepatocyte plasma membrane. The isolation of two organ-specific proteins from human liver (Meyer zum Buschenfelde and Miescher[117]) was followed by the demonstration of organ-specific antibodies in the sera of a proportion of patients with active chronic hepatitis. In addition, active chronic hepatitis has been induced in rabbits by repeated immunization with extracts containing these liver specific proteins. Cellular hypersensitivity, as measured by leucocyte migration inhibition to these proteins was found in 69 percent of 16 patients with active chronic hepatitis, and 50 percent of 12 patients with primary biliary cirrhosis. More recently, evidence was found of cell-mediated sensitization to hepatitis B surface antigen in all patients with acute hepatitis B and transitory sensitization to liver specific lipoprotein was detected in many of the patients (Lee *et al.*[118]).

These findings are consistent with the hypothesis that a cell-mediated immune response to hepatitis B antigen, present early at the onset of acute hepatitis, is the cause of acute liver damage by a cytotoxic effect on virus-infected cells. If the response to liver-specific lipoproteins persisted, it could be responsible for progression to chronic liver damage. It is also postulated that the progressive liver damage of active chronic hepatitis is due to an autoimmune reaction directed against an hepatocyte surface lipoprotein which is initiated in most cases by infection with hepatitis B virus. Lee *et al.*[118] found evidence of cell-mediated immunity to hepatitis B surface antigen in 62 percent of patients with antigen-negative chronic hepatitis, suggesting a high frequency of previous infection with hepatitis B virus. A cellular response to the antigen was found in the majority of hepatitis B antigen positive patients. Evidence of sensitization to liver-specific lipoprotein was found in more than half of the patients, with a similar frequency in the two groups. These results are in agreement with the hypothesis that infection with hepatitis B virus is important in initiating the disease in many cases of active chronic hepatitis and that sensitization to the liver cell membrane antigen is responsible for perpetuation of the liver injury. Thomson *et al.*[119] demonstrated the killing of isolated rabbit hepatocytes *in vitro* when incubated with lymphocytes from 20 of 22 patients with untreated active chronic hepatitis. Blocking experiments strongly suggest the cytotoxicity is due to an immunological reaction directed at a cell surface antigen.

Thus immunological mechanisms and the presence of antibodies reacting with various tissue components may well be involved in the pathogenesis of liver damage. It therefore may be undesirable to employ preparations of hepatitis B surface antigen which contain host cell components or host proteins for immunization against this infection.

A number of laboratories are investigating the subunit structure of purified hepatitis B surface antigen, and it has been shown that subunits consist of polypeptides. Preparations of both the *ad* and *ay* subtypes were solubilized by treatment with sodium dodecyl sulphate (SDS) and mercaptoethanol. The released protein components were defined by SDS-Acryla-

mide gel electrophoresis and the separated polypeptides were stained with Coomassie blue. Analysis of the 20nm spherical particles of subtype *ad* revealed two major polypeptides of 26,000 and 32,000 molecular weight in addition to three minor components in the molecular weight range of 40,000 to 95,000. A similar profile was obtained with antigen subtype *ay*, although different relative densities of staining were produced. Several glycosylated polypeptides have also been detected, the major glycosylated polypeptide corresponding to a molecular weight of 27,000 and 22,000 respectively. The heat stability of the antigen and its resistance to proteases suggest that the carbohydrate may be either an integral component which stabilizes the antigen structure or that the carbohydrate acts as an antigenic determinant.

It has been shown that if the major portion of serum protein is removed by gel filtration, the technique of electrofocusing separates hepatitis B surface antigen from the remaining unwanted serum proteins and reveals a heterogeneity in the surface properties of the small antigen particles (Howard and Zuckerman[120]). The isoelectric points of the two isolated bands were found to differ according to the nature of the subdeterminants and to share at least one common antigenic determinant, as demonstrated by immune electron microscopy. Comparative electrophoresis of antigen iodinated by the lactoperoxidase and chloramine-T methods revealed that at least three polypeptide components can be resolved only with difficulty by electrophoresis but they were found to segregate, when subjected to isoelectric focusing, into two populations of small 20nm particles. An acidic component contained a slower moving polypeptide with a molecular weight of 90,000 and the more basic component contained a polypeptide with a molecular weight of 82,000.

Three major phospholipids have been extracted from the small 20nm antigen particles as well as two glycolipids. Other studies have shown that disulphide linkages play an important role in maintaining the antigenic integrity of the surface antigen. Purified hepatitis B surface antigen labelled with ^{125}I was found to contain six distinct polypeptides with molecular weights ranging from 10,000 to 39,000.

More recently, several of the major polypeptides derived from antigen with determinants *adw* and *ayw* have been isolated and purified. Antisera to these subunits have been produced in guinea pigs. The major polypeptides elicit a vigorous antibody response, and the subunits tested contained the *a, d* or *y* antigenic determinants (Dreesman *et al.*[121]). Shih and Gerin[122] also demonstrated that the structural polypeptides of the surface antigen were immunogenic in guinea pigs. Each polypeptide was found to contain within its structure the group-specific surface antigen determinant *a*. Work is now in progress to determine if such preparations could be used as hepatitis B vaccines.

Perhaps one of the most interesting prospects for the future is the development of a synthetic vaccine. An immunochemical study of purified hepatitis B surface antigen is essential for this project. Analogous to the TMVP decapeptide the primary sequencing of the haptenic peptide of hepatitis B antigen would be the approach for developing a synthetic peptide, which when coupled to a macromolecular carrier, could serve as a suitable immunogen. Once detailed data are available on the protein, peptide and amino-acid composition of this antigen, it should be possible to define by animal immunization the moiety responsible for the antigenic activity. Provided a sufficiently small fragment of the molecule would be immunogenic, then a synthetic vaccine may be feasible (Zuckerman and Howard[111]).

Hepatitis virus C

Viral hepatitis now constitutes the main hazard of the transfusion of blood and certain blood products. For example, it was estimated in 1972 that tranfusion-associated hepatitis caused, in the United States, more than 30,000 cases of overt hepatitis and 1500 to 3000 deaths every year. Since there are many subclinical cases of viral hepatitis the actual incidence in the United States has been estimated as high as 150,000 cases annually.

The discovery of hepatitis B surface antigen and its association with infection has led to the development of a bewildering array of tests for screening blood donors and blood products. It was soon firmly established that blood containing this antigen was associated with a high risk of hepatitis and that such blood should not be used for transfusion. However, it rapidly became apparent that the elimination of antigen-positive blood reduced post-transfusion hepatitis type B by about 30 percent only in some areas.[123] Although the more recent introduction of sensitive methods of detection such as radioimmunoassay reduced further the incidence of post-transfusion hepatitis, cases of hepatitis B and non-B infections continue to occur. These remaining infections are generally ascribed to still insufficiently sensitive assay techniques for hepatitis B (Hollinger *et al.*[124,125]) and to hepatitis A, which can also be transmitted by blood.

The identification by immune electron microscopy of virus-like particles in fecal extracts of patients with hepatitis A and in experimentally infected marmosets and chimpanzees, provided an antigen which could be used for serological tests for hepatitis A antibodies and thus for evidence of infection. Immune electron microscopy has been used widely but immune adherence hemagglutination, complement fixation and radioimmunoassay techniques have been developed and are being applied on a limited scale. Sera from patients with hepatitis B-negative post-transfusion hepatitis in the United States were recently examined for evidence of infection with hepatitis A virus, and although relatively few patients have been tested, seroconversion to hepatitis A was not found in any of these patients (Feinstone *et al.*[126]). A new term was coined; non-A: non-B hepatitis. Feinstone and his colleagues were unable to implicate infection with cytomegalovirus or Epstein-Barr virus, which are known to induce liver damage as part of the generalized infection caused by these herpes viruses. Recently, Alter *et al.*[127] pointed out that in the United States when blood obtained from volunteer donors is pretested by radioimmunoassay for hepatitis B surface antigen, approximately 90 percent of the remaining cases of post-transfusion hepatitis are serologically unrelated either to hepatitis A or hepatitis B viruses. Cytomegalovirus and Epstein-Barr virus were not implicated in non-A: non-B hepatitis and it was considered, therefore, that a previously unrecognised human hepatitis virus may exist. This agent may be hepatitis virus C, a term introduced by Prince *et al.*[128]. They noted that an agent other than hepatitis B was the cause of 71 percent of 51 cases of post-transfusion hepatitis identified during a prospective serological study of 204 patients in New York. The incubation period was relatively long and the clinical and epidemiological features of the infection were not consistent with hepatitis A. It is evident from these studies that a hepatitis virus C may indeed exist, although the application of a battery of modern virological techniques has defied so far the precise criteria which would be virologically acceptable for a new infective agent.

Looking into the future

It is expected that by the beginning of the 21st century a safe hepatitis B vaccine will be available for the immunization of groups at a special risk. The position with hepatitis A is less certain. However, since a candidate hepatitis A virus can now be morphologically and serologically identified, rapid progress may be expected. Although liver cell and organ cultures and cultures of human small intestine have been used extensively, other approaches now seem feasible. These include such techniques as cell fusions and the induction of a cell-permissive state through the use of nucleic acid analogues and other material. Once this major step has been accomplished, because the virus appears to be a simple cubic structure, it should then be a relatively easy matter to produce either a killed or an attenuated live vaccine. This would obviate the present wide scale administration of pooled human immunoglobulin for passive protection against this infection.

It would not be surprising if hepatitis virus C will be fully characterized, but other hepatitis viruses may emerge. To quote Chesterton: "Progress is the mother of problems."

ACKNOWLEDGEMENT

The work in progress at the London School of Hygiene and Tropical Medicine is supported by generous grants from the Medical Research Council, The World Health Organization, The Department of Health and Social Security, The Wellcome Trust and the Wolfson Foundation.

REFERENCES

1. **Cockayne EA:** Catarrhal jaundice, sporadic and epidemic, and its relation to acute yellow atrophy of the liver. *Quart J Med 6:1, 1912*
2. **Bamberger:** Krankheiten des chylopoetischen Systems. *Erlangen, Virchow's Handbuch der Pathologie und Therapie, 5*
3. **Virchow R:** Uber das Vorkommen und den Nachweis des Hepatogenen, insbesondere des Katarrhalischen Icterus. *Virchow Arh Path Anat 32:117, 1865*
4. **Frohlich C:** Uber Icterusepidemien. *Dtsch Arch Klin Med 24 394, 1879*
5. **Eppinger H:** Ikterus. *Wien Klin Wochenschr 21:480, 1908*
6. **Eppinger H:** Die pathogene des Ikterus. *Ergeb Inn Med Kinderheilkd 1:107, 1908*
7. **Von Bormann F, Bader RE, Deines H, Unholtz K:** Hepatitis Epidemica in Deutschland. *Beitr Hyg Epidemiol (Leipzig), Heft 1, 1943*
8. **Monro D:** An account of diseases which were most frequent in British military hospitals in Germany from January 1761 to the return of the troops in England in March 1763. *London, Millar and Cadell, 1764*
9. **Gutzeit K:** Die Hepatitis epidemica. *Munch Med Wochenschr 92:1161 and 1295, 1950*
10. **Cullinan ER:** History of the Second World War, *U.K. Medical Series, Medicine and Pathology*. Ed. V.Z. Cope, London, HMSO, 1952. pp. 230
11. **Stokes W.:** *Lond Med Surg J 5:198, 1839*
12. **Heitler M.:** *Wein Klin Wochenschr 37:957, 1887*
13. **Flindt N.:** *Bibl Laeger 7:420, 1890*
14. **Martin CJ:** Concerning the pathology and aetiology of the infectious jaundice common at the Dardanelles, 1915. *J Army Med Corps 30:102, 1918*
15. **McDonald S:** Acute yellow atrophy. *Edin Med J. 15:208, 1918*
16. **McDonald S:** Acute yellow atrophy in syphilis. *Brit Med J 1:76, 1918*
17. **Stokes JH, Ruedemann R, Lemon WS:** Epidemic infectious jaundice and its relation to the therapy of syphilis. *Arch Int Med 26:521, 1920*
18. **Bergstrand H:** Ueber die akute und chronische Gelbe Leberatrophie. *Leipzig, Georg Thieme, 1930*
19. **Findlay GM, Dunlop JL, Brown HC:** Observations on epidemic catarrhal jaundice. *Trans R Soc Trop Med Hyg 25:7, 1931*
20. **Findlay GM, Dunlop JL:** A fatal case of acute

necrosis of the liver associated with epidemic catarrhal jaundice. *Brit Med J 1:652, 1932*

21. **Findlay GM, MacCallum FO, Murgatroyd F:** Observations bearing on the aetiology of infective hepatitis (so-called epidemic catarrhal jaundice). *Trans R Soc Trop Med Hyg 32:575, 1939*

22. **Lurman:** Eine Icterusepidemie. *Berlin Klin Wochenschr 22:20, 1885*

23. **MacCallum FO:** Historical perspectives. *Can Med Assoc J 106:423, 1972*

24. **Medical Research Council:** Infective hepatitis. *Spec Rep Ser No 66, London, HMSO, 1922*

25. **Murray DH:** Acriflavine, its use, by intravenous injection, in treatment. *J Army Med Corps 54:19, 1930*

26. **Ruge H:** Die Zusammenhange zwischen Syphilis, Salvarsan und der sog Katarrhalischen Gelbsucht auf Grund von 2500 in der Marine von 1919-1929 beobachteten Fallen. *Dermatol Wochenschr 94:278, 1932*

27. **Soffer LJ:** Postarsphenamine jaundice. *Am J Syph Gonorrhoea Vener Dis 21:309, 1937*

28. **Bigger JW:** Jaundice in syphilitics under treatment. *Lancet 1:457, 1943*

29. **Flaum A, Malmros H, Persson E:** Eine mosocomiale Ikterus Epidemic. *Acta Med Scand Suppl 16:544, 1926*

30. **Sherwood PM:** Outbreak of syringe-transmitted hepatitis with jaundice in hospitalized diabetic patients. *Ann Intern Med 33:380, 1950*

31. **Droller H:** An outbreak of hepatitis in a diabetic clinic. *Brit Med J 1:623, 1945*

32. **Hartfall SJ, Garland HG, Goldie W:** Gold treatment of arthritis; review of 900 cases. *Lancet 2:784, 1937*

33. **MacCallum FO:** Jaundice in syphilitics. *Brit J Vener Dis 19:63, 1943*

34. **MacNalty AS:** Acute infectious jaundice and administration of measles serum. *Rep Chief Med Off Minist Health, London, HMSO, 1937, 1938*

35. **Propert SA:** Hepatitis after prophylactic serum. *Brit Med J 2:677, 1938*

36. **Findlay GM, MacCallum FO:** Note on acute. hepatitis and yellow fever immunization. *Trans R Soc Trop Med Hyg 31:297, 1937*

37. **JAMA:** Jaundice following yellow fever vaccination. *119:1110, 1942*

38. **Sergiev PG et al:** Virus Jaundice: epidemic hepatitis in relation to immunization with human serum. *Terap Arkh 18:595, 1940.* **Cited by MacCallum FO, Bauer DJ:** *Lancet 1:622, 1944*

39. **Morgan HV, Williamson DAJ:** Jaundice following administration of human blood products. *Brit Med J 1:750, 1943*

40. **Spurling N, Shone J, Vaughan J:** Incidence, incubation period and symtomatology of homolgous serum jaundice. *Brit Med J 2:409, 1946*

41. **Beeson PB:** Jaundice occurring one to four months after transfusion of blood or plasma. *JAMA 121:1332, 1943*

42. **Beeson PB, Chesney G, McFarlan AM:** Hepatitis following injection of mumps convalescent plasma. *Lancet 1:814, 1944*

43. **McFarlan AM, Chesney G:** Hepatitis following injection of mumps convalescent plasma; reports from American Red Cross-Harvard Field Hospital Unit; epidemiology of hepatitis. *Lancet 1:816, 1944*

44. **Zuckerman AJ:** The History and epidemiology of infectious and serum hepatitis. Virus Diseases of the Liver. *London, Butterworths, 1970, pp. 16-42*

45. **Zuckerman AJ:** Post-transfusion hepatitis. Human viral Hepatitis. *Amsterdam, North Holland-American Elsevier, 1975, pp. 207-224*

46. **Voeght H:** Zur aetiologie der hepatitis epidemica. *Munch Med Wochenschr 89:76, 1942*

47. **Cameron JDS:** Infective hepatitis. *Quart J Med NS 12:139, 1943*

48. **MacCallum FO, McFarlan AM, Miles JAR, Pollock MR, Wilson C:** Infective hepatitis. Studies in East Anglia during the period 1943-47. *Spec Rep Ser Med Res Coun No 273, London, HMSO, 1951*

49. **Havens Jr WP:** The etiology of infectious hepatitis. *JAMA 134:653, 1947*

50. **Havens Jr WP:** Viral hepatitis. *Postgrad Med J 39:212, 1963*

51. **MacCallum FO:** Homologous serum hepatitis. *Lancet 2:691, 1947*

52. **World Health Organization** *Tech Rep Ser No 512, 1973*

53. **Krugman S, Giles JP, Hammond J:** Infectious hepatitis. Evidence for two distinctive clinical, epidemiological and immunological types of infection. *JAMA 200:365, 1967*

54. **Krugman S, Giles JP, Hammond:** Viral hepatitis type B (MS-2 strain). Studies on active immunization. *JAMA 217:41, 1971*

55. **Neefe JR, Stokes Jr J, Reinhold JG, Lukens FDW:** Hepatitis due to the injection of homologous blood products in human volunteers. *J Clin Invest 23:836, 1944*

56. **Paul JR, Havens Jr WP, Sabin AB, Philip CB:** Transmission experiments in serum jaundice and infectious hepatitis. *JAMA 128:911, 1945*

57. **Stokes Jr J, Berk JE, Malamut LL, Drake ME, Barondess, JA, Bashe WJ, Wolman IJ, Farquhar JD, Bevan B, Drummond RJ, Maycock W d' A, Capps RB, Bennett AM:** The carrier state in viral hepatitis. *JAMA 154:1059, 1954*

58. **Neefe JR, Norris RF, Reinhold JG, Mitchell CB, Howell DS:** Carriers of hepatitis virus in the blood and viral hepatitis in whole blood recipients. I. Studies on donors suspected as carriers of hepatitis virus and as sources of post-transfusion hepatitis. *JAMA 154:1066, 1954*

59. **Murray R, Deifenbach WCL, Geller H, Leone NC, Ratner F:** The problem of reducing the danger of serum hepatitis from blood and blood products. *NY State J Med 55:1145, 1955*

60. **Zuckerman AJ, Taylor, PE:** Persistence of the SH-Australia antigen for many years. *Nature 223:81, 1969*

61. **Murray R, Ratner F, Leone NC, Oliphant JW:** Carriers of hepatitis virus in blood and viral hepatitis in whole blood recipients. II. Confirmation of carrier state by transmission experiments in volunteers. *JAMA 154:1072, 1954*

62. **Capps RB, Bennett AM, Stokes Jr J:** A prolonged outbreak of infectious hepatitis in nurses due to a group of small children serving as a

reservoir of the virus. *J Clin Invest 29:802, 1950*

63. **Zuckerman AJ:** The problem and control of hepatitis B infection in the fetus and the newborn. *Infections of the Fetus and the Newborn.* Eds. S. Krugman and A. Gershon, New York, Alan Liss, 1975, pp. 97-113

64. **Zuckerman AJ:** Attempts to isolate the hepatitis virus by tissue culture methods. *Virus Diseases of the Liver,* London, Butterworths, 1970, pp.46-58

65. **Essen KW, Lembke A:** Zur aetiologie der Hepatitis epidemica. *Med Zeitung 1:99, 1944*

66. **Essen KW, Lembke A:** Kann der Erreger der Hepatitis epidemica als identifiziert gelten? *Klin Wschr 27:749, 1949*

67. **Henle W, Drake M, Henle G, Stokes Jr J:** A skin test for infectious hepatitis. *Proc Soc Exp Biol Med 73:603, 1950*

68. **Rightsel WA, Keltschr RA, Tekushan FM, McClean Jr IW:** Tissue-culture cultivation of cytopathogenic agents from patients with clinical hepatitis. *Science 124:226, 1956*

69. **Rightsel WA, Taylor AR, Boggs JD, McClean Jr IW:** Status report on tissue-culture cultivated hepatitis virus. I.Virology laboratory studies. *JAMA 177:671, 1961*

70. **Boggs JD, Capps RB, Weiss CF, McClean Jr IW:** Status report on tissue-culture cultivated hepatitis virus. II. Clinical trials. *JAMA 177:678, 1961*

71. **Cole RA:** Viral agents from patients with infective hepatitis. *Aust J Exp Biol Med Sci 43:497, 1965*

72. **Cross GF, Marmion BP:** Cell culture and infectious hepatitis. *Med J Aust 2:1214, 1966*

73. **Hillis WD:** An outbreak of infectious hepatitis among chimpanzee handlers at a United States Air Force base. *Am J Hyg 73:316, 1961*

74. **Hillis WD:** Viral hepatitis associated with subhuman primates. *Transfusion 3:445, 1963*

75. **Davis EV:** Isolation of viruses from children with infectious hepatitis. *Science 133:2059, 1961*

76. **Hatch MH, Siem RA:** Viruses isolated from children with infectious hepatitis. *Am J Epidemiol 84:495, 1966*

77. **Zuckerman AJ, Dunkley LJ, Love GJ:** Infection of human liver cell cultures with the San Carlos viruses. *Brit J Exp Path 49:235, 1968*

78. **Zuckerman AJ, Bird RG, Dunkley LJ, Love GJ:** Examination by electron microscopy of the San Carlos viruses isolated from children with infectious hepatitis. *J Clin Path 22:40, 1969*

79. **Zuckerman AJ:** Tissue and organ culture studies of hepatitis B virus. *Am J Med Sci 270:205, 1975*

80. **Havens Jr WP:** Review of attempts to test for immune bodies produced against the hepatitis agent. Symposium on the laboratory propagation and detection of the agent of hepatitis. *Natl Acad Sci, Natl Res Counc Publication 322, 1954,* pp. 93-101

81. **Gear JHS:** Discussion. In: *Proceedings of the Fourth International Congress on Tropical Medicine and Malaria, Washington. 1:482, 1948*

82. **Gear JHS:** Cited by Zuckerman AJ. *Human Viral Hepatitis.* Amsterdam, North Holland-American Elsevier, 1975, p.14

83. **Sawyer WA, Meyer KF, Eaton MD, Bauer JH, Putnam P, Schwentker FF:** Jaundice in army personnel in the western region of the United States and its relation to vaccination against yellow fever. *Am J Hyg 39:337 and 40:35, 1944*

84. **Eaton MD, Murphy WD, Hanford VL:** Heterogenetic antibodies in acute hepatitis. *J Exp Med 79:539, 1944*

85. **Pollard M, Bussell RH:** Rapid laboratory diagnostic procedures in microbiology: complement-fixation reaction in serum hepatitis. *USAF School of Aviation Medicine, Project No. 21-1401-0005, Report No. 3, 1953*

86. **Allison AC, Blumberg BS:** An isoprecipitation reaction distinguishing human serum protein types. *Lancet 1:634, 1961*

87. **Blumberg BS, Alter HJ, Visnich S:** A "new" antigen in leukemia sera. *JAMA 191:541, 1965*

88. **Blumberg BS:** Australia antigen, hepatitis and leukemia. *Tokyo J Med Sci 76:1, 1968*

89. **Blumberg BS, Gerstley BJS, Hungerford DA, London WT, Sutnick AI:** A serum antigen (Australia antigen) in Down's syndrome, leukemia and hepatitis. *Ann Intern Med 66:924, 1967*

90. **Sutnick AI, London WT, Gerstley BJS, Cronlund MM, Blumberg BS:** Anicteric hepatitis associated with Australia antigen. Occurrence in patients with Down's syndrome. *JAMA 205:670, 1968*

91. **Deinhardt F:** Hepatitis in subhuman primates and the hazards to man. *Infections and Immunosuppression in subhuman Primates.* Eds. H. Balner and W.I.B. Beveridge, Cophenagen, Munksgaard, 1970, pp. 55-63

92. **Deinhardt F, Holmes AW, Capps RB, Popper H:** Studies on the transmission of human viral hepatitis to marmoset monkeys. I. Transmission of disease, serial passages and description of liver lesions. *J. Exp Med 125:673, 1967*

93. **Holmes AW, Wolfe L, Deinhardt F, Conrad ME:** Transmission of human hepatitis to marmosets: further coded studies. *J infect Dis 124:520, 1971*

94. **Lorenz D, Barker L, Stevens D, Peterson M, Kirchstein R:** Hepatitis in the marmoset, *Saguinus mystax. Proc Soc Exp Biol Med 135:348, 1970*

95. **Holmes AW, Deinhardt F, Wolfe L, Froesner G, Peterson D, Casto B, Conrad ME:** Specific neutralization of human hepatitis type A in marmoset monkeys. *Nature 243:419, 1973*

96. **Mascoli CC, Ittensohn D, Villarejos VM, Arguedas, JA, Provost PJ, Hilleman MR:** Recovery of hepatitis agents in the marmoset from human cases occurring in Costa Rica. *Proc Soc Exp Biol Med 142:276, 1973*

97. **Provost PJ, Ittensohn OL, Villarejos VM, Arguedas JA, Hilleman MR:** Etiologic relationship of marmoset-propagated CR 326 hepatitis A virus to hepatitis in man. *Proc Soc Exp Biol Med 142:1257, 1973*

98. **Holmes AW, Wolfe L, Rosenblate H, Deinhardt F:** Hepatitis in marmosets: induction of disease with coded specimens from a human volunteer study. *Science 165:816, 1969*

99. **Deinhardt F, Wolfe L, Junge U, Holmes**

AW: Viral hepatitis in nonhuman primates. *Can Med Assoc J 106:468, 1972*

100. **Zuckerman AJ:** Hepatitis in nonhuman primates. *Human Viral Hepatitis*. Amsterdam, North Holland-American Elsevier, 1975, pp. 386-394

101. **Maynard JE, Berquist KR, Drushak DH, Purcell RH:** Experimental infection of chimpanzees with the virus of hepatitis B. *Nature 237:514, 1972*

102. **Barker LF, Chisari FV, McGrath PP, Dalgard DW, Kirschstein RL, Almeida JD, Edginton TS, Sharp DG, Peterson MR:** Transmission of type B viral hepatitis to chimpanzees. *J. Infect Dis 127:648, 1973*

103. **Zuckerman AJ:** Electron microscopy and immune electron microscopy. *Bull WHO 42:975, 1970*

104. **Almeida JD, Rubenstein D, Stott EJ:** New antigen-antibody system in Australia antigen-positive hepatitis. *Lancet 2:1225, 1971*

105. **Harris WW, Anderson NG, Bartlett TW, Rutenberg EL, McCauley LL, Kniseley RM:** Unusual particles in human plasma from leukemia and lymphosarcoma. *Natl Cancer Inst Monogr 21:389, 1966*

106. **Magnius LO, Espmark A:** New specificities in Australia antigen-positive sera distinct from the Le Bouvier determinants. *J Immunol 109:1017, 1972*

107. **Feinstone SM, Kapikian AZ, Purcell RH:** Hepatitis A: Detection by immune electron microscopy of virus-like antigen associated with acute illness. *Science 182:1026, 1973*

108. **Almeida JD, Deinhardt F, Zuckerman AJ:** Virus-like particles in hepatitis A-positive fecal extracts. *Lancet 2:1083, 1974*

109. **Thornton A, Zuckerman, AJ, Almeida JD:** Chimpanzee model for hepatitis A virus. *Lancet 2:226, 1975*

110. **Zuckerman AJ, Howard CR:** Prospects for hepatitis B vaccines. *Nature 246:445, 1973*

111. **Zuckerman AJ, Howard CR:** Towards hepatitis B vaccines. *Bull NY Acad Med 51:491, 1975*

112. **Soulier JP, Blatix C, Courouce AM, Benamon D, Amouch P, Drouet J:** Prevention of virus B hepatitis (SH hepatitis). *AM J Dis Child 123:429, 1972*

113. **Purcell RH, Gerin JL:** Hepatitis B subunit vaccine: a preliminary report of safety and efficacy tests in chimpanzees. *AM J Med Sci 270:395, 1975*

114. **Neurath AR, Prince AM, Lippin A:** Hepatitis B antigen: antigenic sites related to human serum proteins revealed by affinity chromatography. *Proc Nat Acad Sci 71:2663, 1974*

115. **Charles R, Steiner S, Melnick JL, Dreesman GR:** Glycoproteins associated with hepatitis B antigen. *Intervirology 1:224, 1973*

116. **Steiner S, Huebner MT, Dreesman GR:** Major polar lipids of hepatitis B antigen preparations: evidence for the presence of a glycosphingolipid. *J Virol 145:572, 1974*

117. **Meyer zum Buschenfelde KH, Kossling FK, Miescher PA:** Experimental chronic active hepatitis in rabbits following immunization with human liver proteins. *Clin Exp Immunol 11:99, 1972*

118. **Lee, WM, Reed WD, Mitchell CG, Galbraith RM, Eddleston ALWF, Zuckerman AJ, Williams R:** Cellular and humoral immunity to hepatitis B surface antigen in active chronic hepatitis. *Brit Med J 1:705, 1975*

119. **Thomson AD, Cochrane MAG, McFarlane IG, Eddleston ALWF, Williams R:** Lymphocyte cytotoxicity to isolated hepatocytes in chronic active hepatitis. *Nature 252; 721, 1974*

120. **Howard CR, Zuckerman AJ:** Electrofocusing of hepatitis B antigen. *J Gen Virol 20:253, 1973*

121. **Dreesman GR, Chairez R, Suarez M, Hollinger FB, Courtney RJ, Melnick JL:** Production of antibody to individual polypeptides derived from purified hepatitis B surface antigen. *J Virol 16:508, 1975*

122. **Shih JWK, Gerin JL:** Immunochemistry of hepatitis B surface antigen (HBsAg): preparation and characterization of antibodies. *J Immunol 115:634, 1975*

123. Viral hepatitis. *World Health Org Tech Rep, Ser no. 512, 1973*

124. **Hollinger FB, Aach RD, Gitnick GL, Roche JK, Melnick JL:** Limitations of solid-phase radioimmunoassay for HBAg in reducing frequency of post-transfusion hepatitis. *N Engl J Med 289:385, 1973*

125. **Hollinger FB, Werch J, Melnick JL:** A prospective study indicating that double-antibody radioimmunoassay reduces the incidence of post-transfusion hepatitis. *New Engl J Med 290:1104, 1974*

126. **Feinstone SM, Kapikian AZ, Purcell RH, Alter HJ, Holland PV:** Transfusion-associated hepatitis not due to viral hepatitis type A or B. *New Engl J Med 292:767, 1975*

127. **Alter HJ, Holland PV, Purcell RH:** The emerging pattern of post-transfusion hepatitis. *Am J Med Sci 270:329, 1975*

128. **Prince AM, Brotman B, Grady GF, Kuhns WJ, Hazzi C, Levine RW, Millian SJ:** Long-incubation post-transfusion hepatitis without serological evidence of exposure to hepatitis B virus. *Lancet 2:241, 1974*

RADIOIMMUNE ASSAY FOR ANTI-CORE AS EVIDENCE FOR EXPOSURE TO HEPATITIS B VIRUS

L. R. Overby

C. M. Ling

There is much evidence suggesting that the hepatitis B virus could be highly defective in assembly of infectious virions, thereby giving rise to relatively high quantities of virus-induced products in infected cells.[1] Hepatitis B antigen(HBsAg) in blood serum has been a consistent marker for the presence of an infectious agent.[2,3] The HBsAg resembles a virus morphologically, but there has been no consistent evidence for infectivity or nucleic acid associated directly with this particle.[4] The HBsAg is immunogenic since anti-HBs is normally found in patients and animals during recovery and convalescence.[5,6] The presence of anti-HBs is associated with protective immunity,[7,8] and this suggests that HBsAg is a structural component of the infectious virion. Thus, the association of HBsAg as a marker for infectivity of blood and blood products, and anti-HBs as a marker for immunity has stimulated widespread diagnostic and epidemiologic studies of hepatitis type B. Highly sensitive and specific immunologic assay systems for antigen and antibody have been necessary and useful reagents for these studies.[9]

Awareness of multiple transmission routes and the use of highly sensitive immunologic test procedures for HBsAg have been reported to significantly reduce transfusion-associated hepatitis type B[10,11] However, in careful studies of recipients of blood negative for HBsAg, Goldfield[3] has reported evidence of exposure to the antigen in 7 of 465 recipients. This suggests that further studies of other immunologic systems associated with the hepatitis B virus could lead to more complete diagnosis of disease and detection of the infectious agent. The Dane particle[12] with its associated core[13] has led to identification of core (HBcAg) and anti-core (Anti-HBc) as a second immunologic system. The finding that the surface of the Dane particle contains HBsAg and the internal core particle contains DNA and DNA polymerase[14-16] has strengthened the proposal for this particle as a candidate for the infectious virus. However, until tissue culture or convenient laboratory animal models are developed, immunochemical, biochemical or electron microscopic techniques are necessary for assessing the presence and possible infectivity of Dane particles.

Two other macromolecular components have been reported in serum of patients or carriers of HBsAg. The e antigen, or its associated anti-e, has been found frequently in serum of patients and carriers of HBsAg.[17] The frequent occurrence of antibody suggests that e antigen is immunogenic. There appears to be little objective evidence as to whether e antigen is of virus or host origin.

In our laboratory studies of the possible association of nucleic acids with HBsAg, we consistently found non-particulate DNA in plasmas of carriers of the antigen.[18] The significance, if

From the Division of Expermental Biology, Abbott Laboratories, North Chicago, Illinois 60064

Lacy R. Overby, PhD., Director of Experimental Biology, Abbott Laboratories

Chung-Mei Ling, Ph.D., Laboratory Head, Biochemistry Laboratories, Abbott Laboratories

any, of this finding is not clear. This linear, double-stranded DNA could be of host or viral origin. There is no evidence that this DNA is immunogenic, although, serum anti-nuclear activity is a frequent finding in pathologic tissue injury.

The above four possible immunologic systems associated with the hepatitis B virus are shown in Fig. 1. Sensitive and specific immunochemical reagents and procedures in each case are required to understand and assess possible diagnostic significance. As discussed below, we have developed reagents for the core/anti-core system and have completed preliminary surveys of blood donor populations.

PURIFICATION OF DANE PARTICLES

Plasmas positive for HBsAg and DNA polymerase[14] were pooled and used as the source of Dane particles. The purification procedure for Dane particles consisted of four major steps: pelleting by centrifugation at 100,000 x g, two cycles of isopycnic banding in sucrose gradients, and a single isopycnic banding in cesium chloride. DNA polymerase activity was used as indicator of Dane particles throughout the purification procedure. Two populations of Dane particles with different buoyant densities were usually present in HBsAg positive plasmas; one

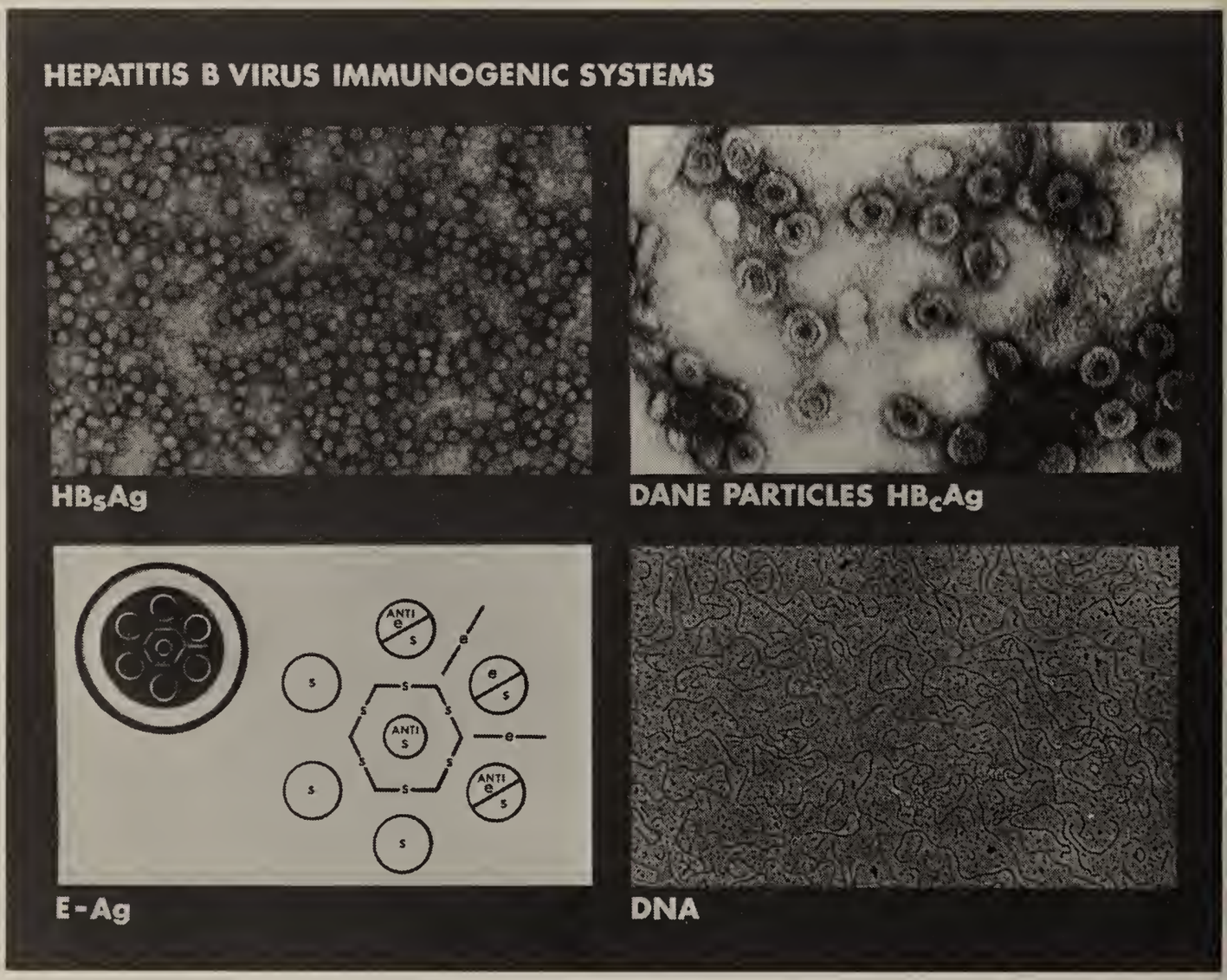


Fig. 1—Antigenic markers of hepatitis B virus. Electron micrographs show 22 nm HBsAg particles (upper left), 42 nm Dane particles with 27 nm cores (upper right), and DNA molecules isolated from HBsAg containing plasma (lower right), At the lower left is a Rheophoresis pattern showing typical e-antigen/anti-e precipitin lines between HBsAg positive samples in the peripheral wells; the center well contains anti-HBs serum and the HBsAg/anti-HBs precipitin lines around the center well show non-identity with the e-antigen/anti-e lines.

banded in sucrose at a density of 1.28 g/cm,[3] and the other at a density of 1.25 g/cm[3] (Fig. 2). In general, the lighter Dane particle band in sucrose gradient was the major of the two, but relative quantities of the two populations in terms of DNA polymerase activities varied from batch to batch of plasma pools. The light and heavy peaks were selected and rebanded in cesium chloride. In electron micrographs, no morphological difference was observed between these two populations of Dane particles with different densities (Fig. 3). The DNA polymerase activities however, did show some differences: the enzyme activity of the lighter Dane particles was enhanced by Nonidet P-40 detergent and 2-mercaptoethanol, while that of the heavier Dane particles was slightly inhibited by the detergent and the reducing agent. These chemicals remove the outer surface of Dane particles.[14,15] Enhancement of polymerase activity is most likely the result of increased penetration of substrates to the enzyme within the core of Dane particles. Lack of enhancement of the enzyme in the heavier particles by Nonidet P-40 and

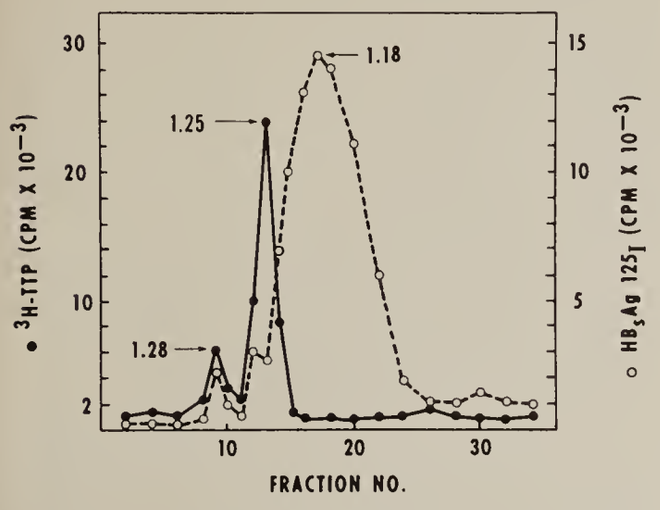


Fig. 2—Distribution of DNA polymerase activity from Dane particle enriched plasma fractions in a linear sucrose gradient. ^{3}H-TTP incorporation - measure of polymerase activity. ^{125}I-cpm - radioimmune assay for HBsAg.

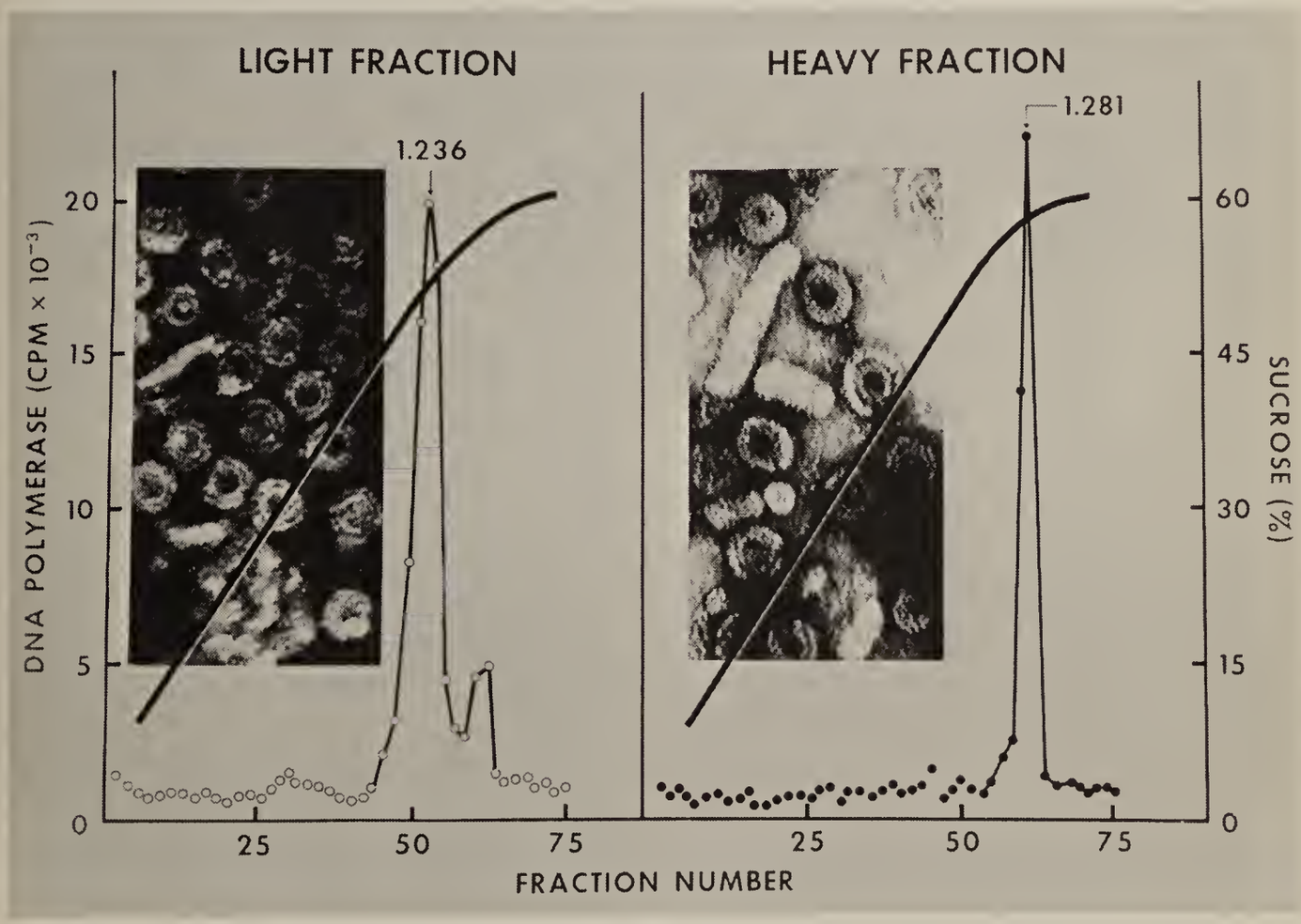


Fig. 3—Repurification of heavy and light DNA polymerase activity by isopycnic banding in CsCl. Peaks of activity were selected from gradients, as in Fig. 2, and centrifuged to equilibrium in CsCl. Electron micrographs show cosedimentation of Dane particles with DNA polymerase at each density.

2-mercaptoethanol suggests an altered structure of these particles resulting in a different density. In general, the numbers of Dane particles of the two fractions per unit volume, estimated by electron microscopy,[19] correlated directly to the levels of DNA polymerase activities.

Purification of Dane particle cores:

Highly purified Dane particles from either heavy or light fraction were treated with Nonidet P-40 and 2-mercaptoethanol to remove surface components.[14,15] The preparations were then mixed with equal volumes of genetron and shaken vigorously to liberate the free cores. DNA polymerase remained in the aqueous phase. Further purification of the aqueous DNA polymerase activity in linear sucrose gradients gave a homogeneous peak with a sedimentation rate of approximately 110 S (Fig. 4). Electron microscopy showed that 27 nm core particles co-sedimented with the polymerase. Only trace amounts of HBsAg were detected in the sucrose gradient by radioimmune assay.

Immunogenicity and antigenicity of Dane particle cores:

Guinea pigs and rabbits were immunized by multiple-site injection with the sucrose gradient purified core preparation mixed with equal volumes of Freund's adjuvant.[20] Serums from these immunized animals were reactive with purified Dane cores and were routinely examined for anti-HBc titres by a direct radioimmuno assay described in a later section of this paper. Guinea pigs were consistently better producers of anti-HBc than were rabbits. The hyperimmune anti-HBc serums were passed through immunoadsorbent columns of purified HBsAg and normal human serum proteins to remove possible antibodies induced by these components. As shown in Fig. 5, the adsorbed serum reacted at a dilution of 8000 with HBcAg, but gave no detectable reaction at any dilution with HBsAg.

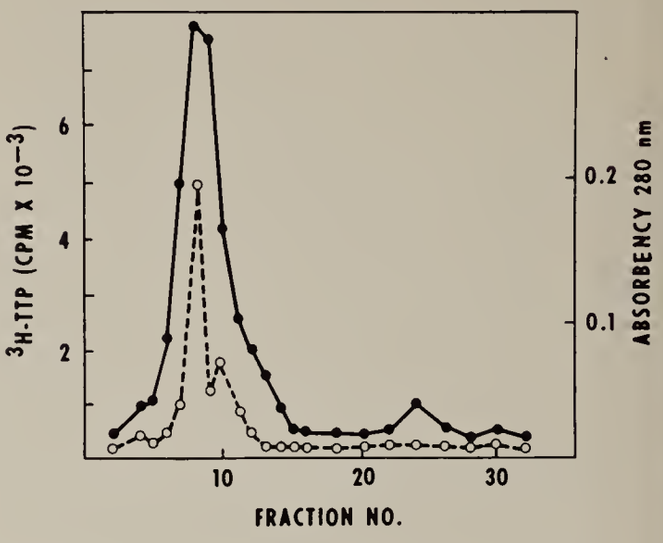


Fig. 4—Purification of Dane cores by rate sedimentation in a linear sucrose gradient. Highly purified Dane particles (2 cycles of sucrose gradient centrifugations and 1 cycle of CsCl isopycnic banding) were treated with 1 percent Nonidet P-40 and 0.3 percent 2-mercaptoethanol for 3 hours. The reaction mixture was extracted with equal volume of genetron and the aqueous phase centrifuged on a 15 to 65 percent linear sucrose gradient in a Spinco SW-27 rotor at 25,000 rpm for 18 hours. One tenth ml of each fraction was assayed for ^{3}H-TTP incorporation into DNA and another 0.1 ml was measured for adsorbency at 280 mm.

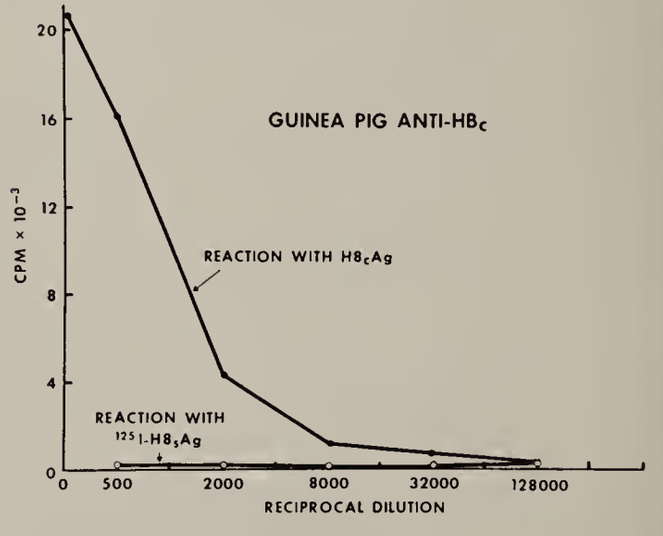


Fig. 5—Specificity of hyperimmune serum of guinea pigs immunized with highly purified Dane cores. A pool of guinea pig hyperimmune serum was circulated through an immunosorbent column of HBsAg and through another with fixed normal human serum to remove anti-HBs and antibodies to normal human serum proteins. The adsorbed serum was serially diluted and assayed for anti-HBc as described in the text and Fig. 6 and for anti-HBs by radioimmune assay (Ausab™).

RADIOIMMUNOASSAYS FOR HBcAG AND ANTI-HBc

Direct test for non-human anti-HBc

Purified HBcAg was bound to polystyrene beads coated by adsorption with a diluted human serum positive for anti-HBc. The assay principle is illustrated in Fig. 6. The serum to be titered was then incubated with the HBcAg-coated bead giving a complex of anti-HBc in the solid phase. The radioactive probe used in a second step was highly purified rabbit anti-guinea pig IgG labeled with ^{125}I-iodine. The resulting count rate of the multiple layered bead was then in direct proportion to anti-HBc in the sample.

Direct test for HBcAg

Anti-HBc coated beads served as the basic reagent for detecting and quantifying HBcAg, as illustrated in Fig. 7. In this case, ^{125}I-labeled hyperimmune guinea pig IgG containing anti-HBc served as the radiolabeled probe. The resulting count rate of the multiple-layer product was in proportion to HBcAg in the specimen.

Competitive test for human anti-HBc

Fig. 8 illustrates the principles used for a solid phase competitive assay for anti-HBc. The solid phase reagent is HBcAg bound to polystyrene beads as in the direct test for guinea pig anti-

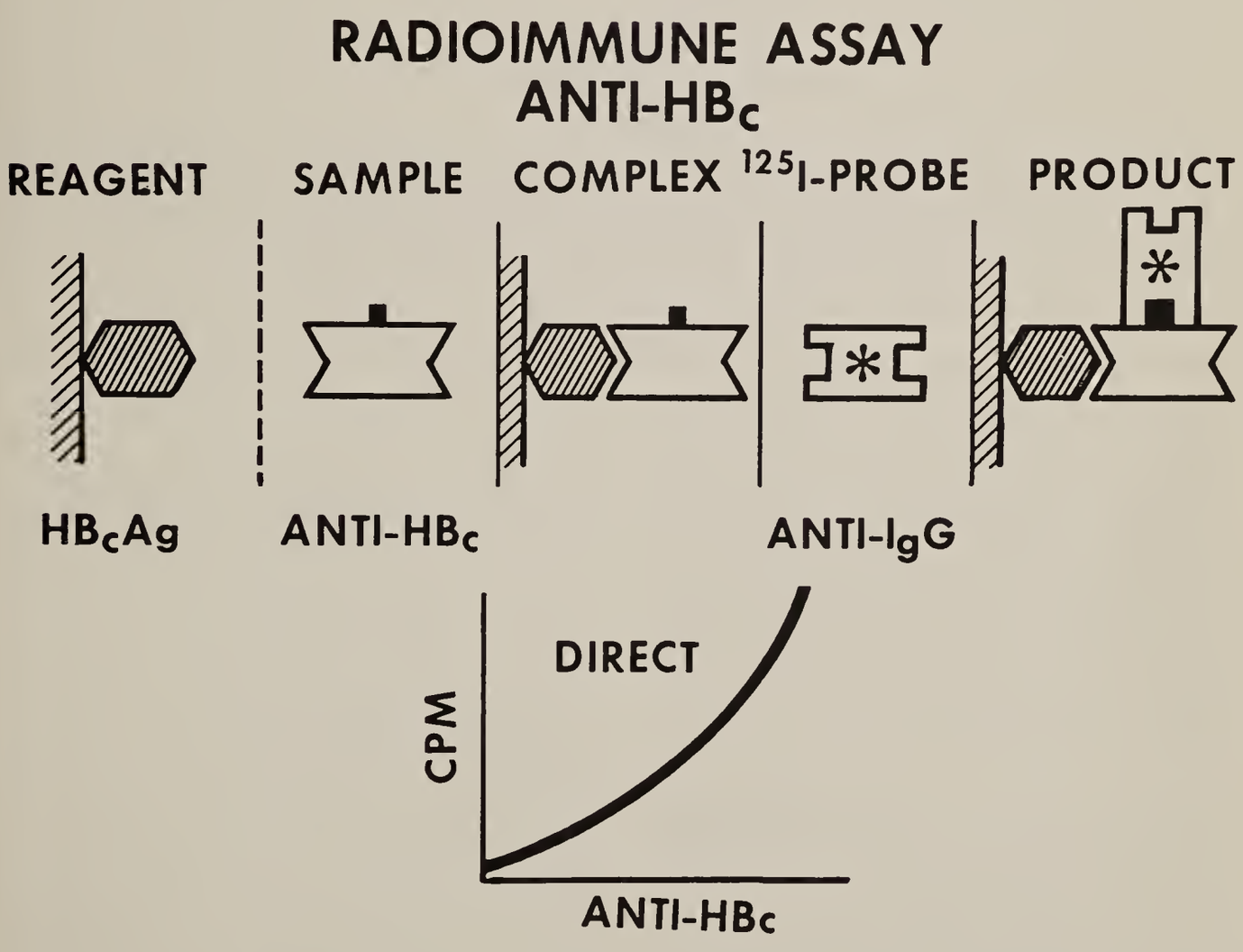


Fig. 6—Schematic illustration of solid-phase direct radioimmuno assay for guinea pig anti-HBc. Polystyrene beads of 6 mm diameter were first coated with a diluted serum positive for anti- HBc and then coated by reaction with purified HBcAg, illustrated as the solid phase reagent. The ^{125}I-probe was immunosorbent purified rabbit antibody against guinea pig IgG, labeled with ^{125}I-iodine. The sample, 0.1 ml of guinea pig anti-HBc serum, diluted in normal human serum, was incubated with the solid phase at 45°C for 2 hours. After washing, the solid phase was incubated with the ^{125}I-antibody in an equal mixture of rabbit serum and human serum at 45°C for another 2 hours. The beads were again washed and counted for ^{125}I-antibody uptake.

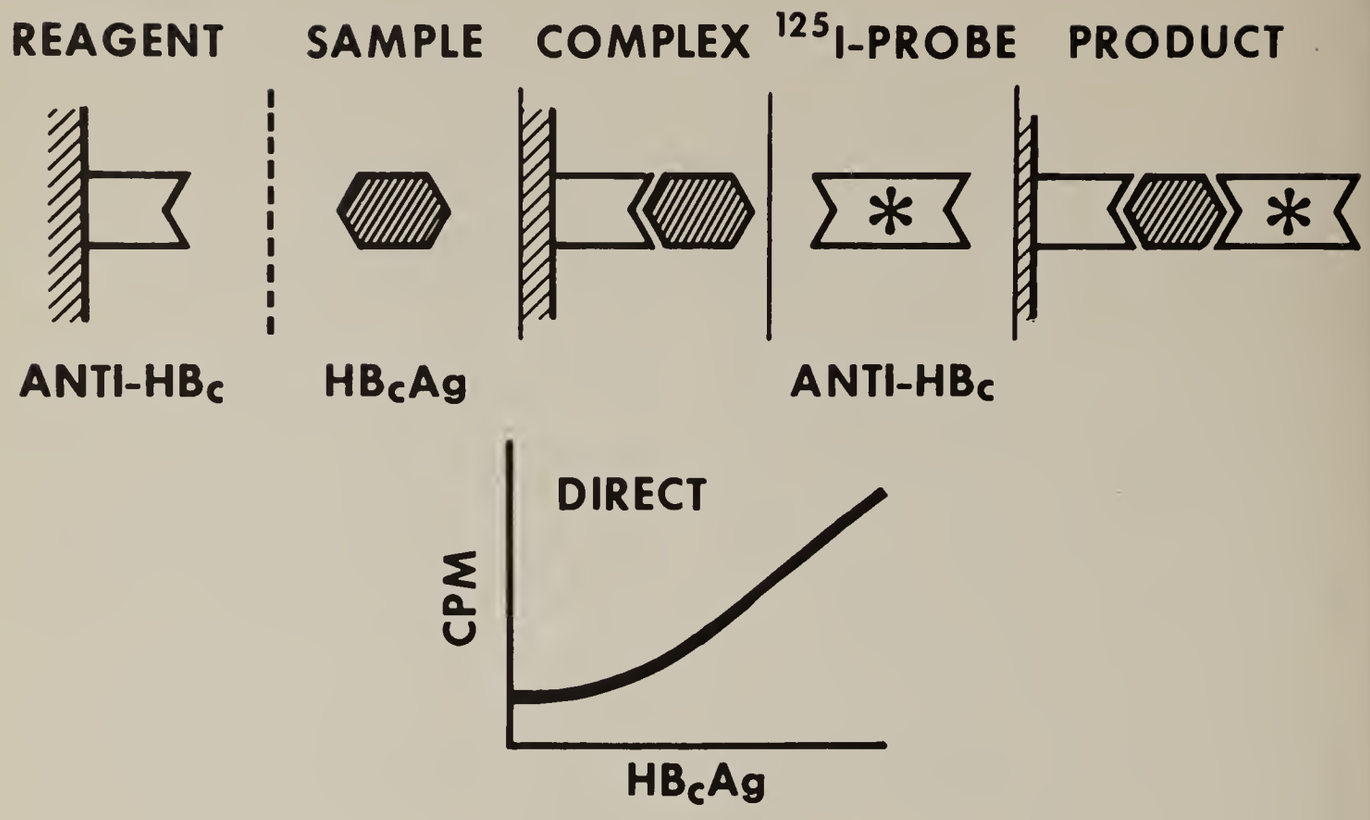


Fig. 7—Schematic illustration of a solid phase direct radioimmuno assay for HBcAg. The solid phase, a 6 mm polystyrene bead, was coated with human anti-HBc serum, and the radiolabeled probe was ^{125}I-iodine labeled guinea pig IgG containing anti-HBc. The assay procedure consists of two incubation steps, 18 hours at room temperature and 2 hours at 45°C respectively, each followed by a washing step.

HBc, above. This reagent is first reacted with an unknown serum containing anti-HBc. The resulting complex is challenged with a constant amount of guinea pig ^{125}I-anti-HBc in a second step. The degree of competition of unknown with labeled probe is an indication of the level of anti-HBc. For the following studies the 50 percent inhibition point was selected as the basis for anti-HBc positivity.

HBcAg activity of Dane cores from chimpanzee liver nuclei and human plasma Dane particles

When highly purified Dane particles from human plasma were treated with Nonidet P-40 detergent and 2-mercaptoethanol, further extracted with genetron, and sedimented on a sucrose gradient, the main peak of HBcAg activity as revealed by radioimmune assay coincided with DNA polymerase activity; nonparticulate HBcAg was also observed at the top of the gradient, indicating fragmentation of the Dane core during the detergent and genetron treatments (Fig. 9). Analysis of the fractions for HBcAg revealed an absence of significant surface antigen in the core particle peak. A sample of chimpanzee liver nuclei extract containing free 27 nm Dane core particles (kindly supplied by Drs. L. Barker and J. Hoofnagle) was centrifuged in a similar linear sucrose gradient and assayed for HBcAg with the immunoglobulins specific for human plasma Dane cores. A homogeneous peak was observed (Fig. 10). No DNA polymerase activity or HBsAg was detectable in any of these fractions. The HBcAg activity of chimpanzee hepatocyte origin showed a higher sedimentation coefficient than

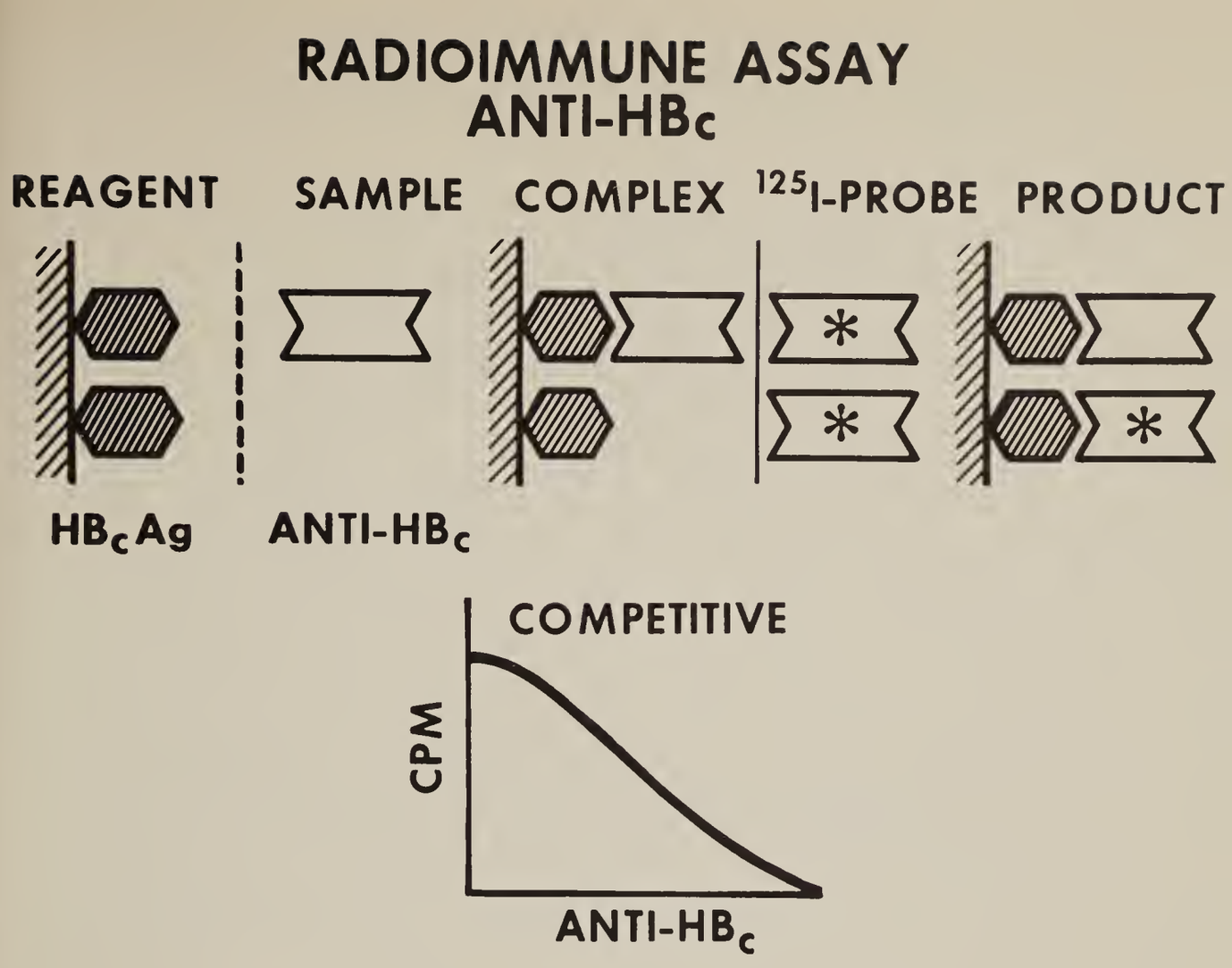


Fig. 8—Schematic illustration of solid phase inhibition (indirect) radioimmunoassay for human anti-HBc. This test uses the same solid phase reagent as in the direct RIA for guinea pig anti-HBc (Fig. 6) and the same radio-indicator for the HBcAg test, ^{125}I-iodine labeled guinea pig anti-HBc (IgG) (Fig. 7). The two incubation steps also followed the same conditions as described in Fig. 6. When the sample contained anti-HBc, the uptake of ^{125}I-anti-HBc by the solid phase is proportionately reduced.

the marker ^{125}I-HBsAg and similar to the 110 S Dane cores from human plasmas.

FREQUENCY DISTRIBUTION OF ANTI-CORE

The above studies suggested reliable specificity and sensitivity for the competitive radioimmune assay for anti-HBc. Serums from consecutive plasmapheresis donors were analyzed for anti-HBc, for HBsAg (Ausria II™, using ^{125}I-anti-HBs), and for anti-HBs (Ausab™, using ^{125}I-HBsAg). Correlation of findings with regard to surface and core systems is summarized in Table I. About 81 percent of the HBsAg positive specimens also contained anti-HBs. Additional studies have suggested that all HBsAg positive serums may contain anti-HBc, detectable with a more sensitive procedure. About 15 percent of the specimens containing anti-HBs also contained anti-HBc. This suggests that the persistence of anti-HBc in these donors may be of shorter duration than persistence of anti-HBs. A third category of 3105 donors showed no evidence of prior exposure to the hepatitis B virus, based on negative results with tests for surface antigen and antibody. However, almost 5 percent of this group were positive for anti-core. Similar results were observed with a group of 1049 volunteer blood donors. The HBsAg

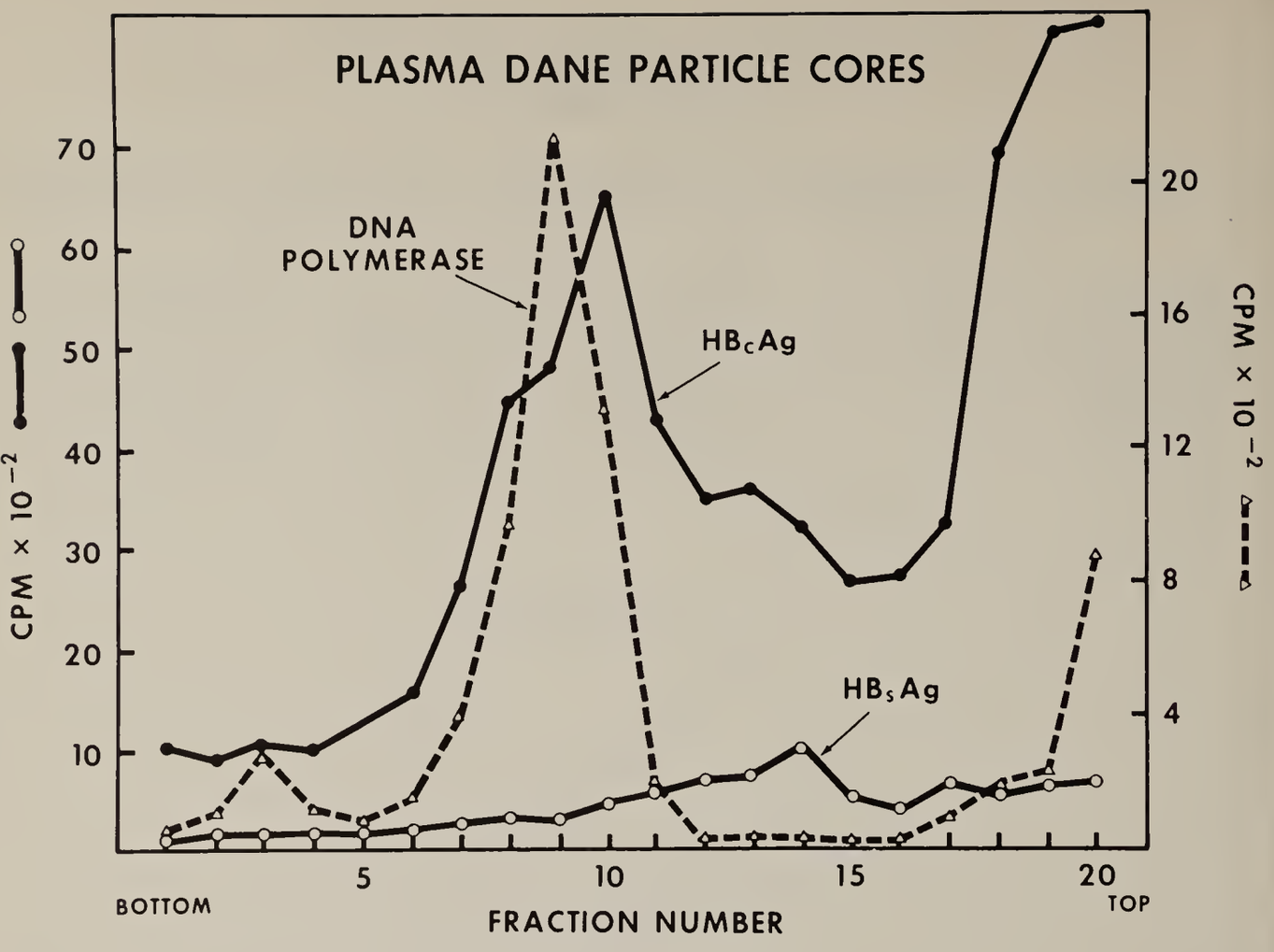


Fig. 9—Sucrose gradient sedimentation pattern of HBcAg after detergent and genetron treatments of Dane particles. The same experimental procedures of Fig. 4 were followed except, in addition, 0.02 μl aliquot of each fraction was diluted to 0.2 ml and assayed for HBcAg by the method described in Fig. 7.

positive serums were not available for testing. About 21 percent of the anti-HBs specimens were also positive for anti-core. About 2 percent of the surface antigen and antibody negative donors were positive for anti-core. In a collection of HBsAg positive serums from carriers, 106 of 113 (94 percent) were positive for anti-HBc.

In the above studies anti-HBc was found in 290 (7.3 percent) of the 3929 plasmapheresis donors, and 34 (3.2 percent) of 1049 volunteer blood donors. There were 145 and 19 donors,

TABLE I

CORRELATION OF ANTI-HBc WITH HEPATITIS B SURFACE ANTIGEN AND ANTIBODY IN DONOR POPULATIONS

	Plasmapheresis Donors			Volunteer Donors			HBsAg Carriers		
	No. Sample	Anti-HBc +	%	No. Sample	Anti-HBc+	%	No. Sample	Anti-HBc +	%
HBsAg +	31	25	80.6	3	NA*		113	106	93.8
Anti-HBs +	793	120	15.1	72	15	20.8			
HBs Neg.	3105	145	4.6	974	19	1.9			
Total	3929	290	7.3	1049	34	3.2	113	106	93.8

*Not available for testing

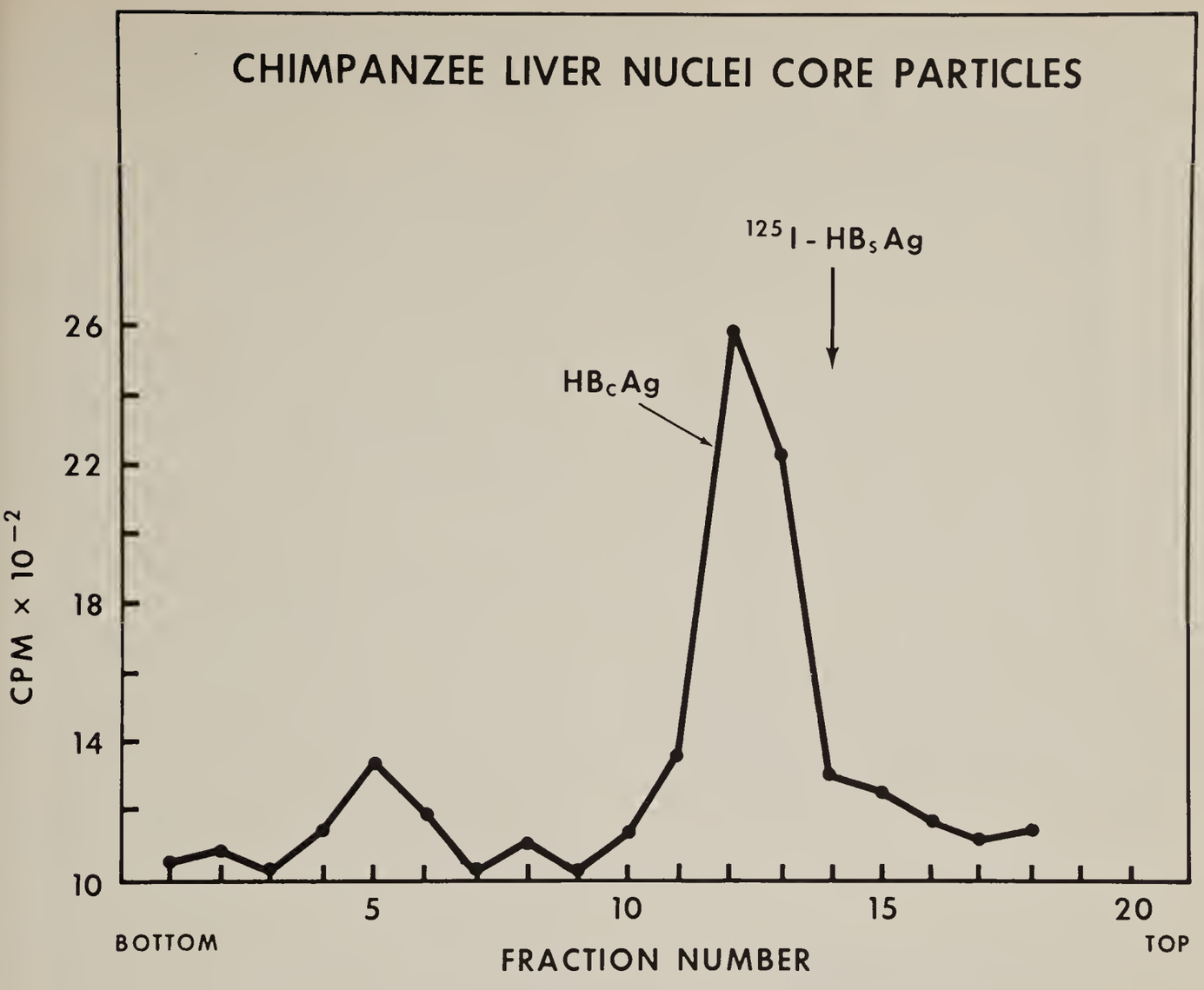


Fig. 10—Sucrose gradient sedimentation and radioimmuno assay for HBcAG from chimpanzee liver cell nuclei extract. The chimpanzee liver cell nuclei extract, previously shown by electron microscopy containing 27 nm naked Dane cores, was sedimented in a linear sucrose gradient under the same conditions described in Fig. 4. Each fraction was assayed for HBcAg as in Fig. 7. The arrow at the left side of the HBcAg peak is the peak fraction of ^{125}I-labeled HBsAg centrifuged in a separate bucket.

respectively, where anti-HBc was the only detectable marker for hepatitis B virus exposure.

These results agree qualitatively with two other studies of anti-HBc occurring in healthy populations. Hoofnagle et al.[21,22] analyzed serums from commercial donors, volunteer donors and HBsAg carriers by complement fixation with HBcAg from infected chimpanzee liver hepatocytes. Anti-HBc was found in 8 of 100 commercial donor serums and 2 of 200 volunteer donor serums. There were 5 and 1 donors, respectively, where anti-HBc was the only evidence of exposure to the hepatitis B virus. Tsuda et al.[23] used immune adherence hemagglutination with purified plasma Dane particle cores for the analyses of the serum from 215 healthy blood donors. There were 36 positive for anti-HBc. These were composed of 2 of 2 HBsAg positives, 28 of 31 anti-HBs positives and 6 from the 192 surface-negative group.

Further proof of specificity and improved sensitivity of the competitive radioimmune procedure is needed. However, it seems clear that analysis of anti-HBc can have epidemiologic and diagnostic significance. Possible interpretations of the presence of anti-HBc and absence of HBsAg or anti-HBs are:

1. Early convalescence from disease with undetectable levels of replicating virus, before the appearance of detectable anti-HBs;
2. A carrier state of HB virus in which the presence of HBsAg is below detectable levels, while anti-HBc is continuously stimulated;
3. Restimulation of the immune system in previously immune individuals where anti-surface has decreased to undetectable levels;
4. Persistence of anti-HBc, in some cases, for periods where anti-HBs has decreased to undetectable levels.

Further understanding of the above possibilities will depend on fully validating the specificity and sensitivity of the immunologic procedures and upon extensive epidemiologic correlations.

REFERENCES

1. **Gerin JL:** Biochemical characterization of Australia antigen, evidence for defective particles of hepatitis B virus. *Am J Pathol 81:651-668, 1975*
2. **Alter HJ, Holland PV, Purcell RH:** The emerging pattern of post-transfusion hepatitis. *AM J Med Sci 270:329, 1975*
3. **Goldfield M, Block HC, Bill J, Srihongse S, Pizzuti W:** The consequences of administering blood pretested for HBsAg by third generation techniques: A progress report. *Am J Med Sci 270:335, 1975*
4. **Gerin JL, Holland PV, Purcell HR:** Australia antigen: large scale purification from human serum and biochemical studies of its proteins. *J Virol 7:569, 1971*
5. **Barker LF, Peterson MR, Schulman NR,** et al.: Antibody responses in viral hepatitis, type B. *JAMA 223:1005, 1973*
6. **Barker LF,** et al.: Transmission of type B viral hepatitis to chimpanzees. *J Infect Dis 127:648, 1973*
7. **Grady GF:** Passive immunization against viral hepatitis-status and prospects. *Am J Med Sci 270:369, 1975*
8. **Desmyter J, Bradburne AF,** et al.: Hepatitis B immunoglobulin in prevention of HBs antigenemia in haemodialysis patients. *Lancet 2:377, 1975*
9. **Miller JP, Overby LR:** Nuclear Medicine *In Vitro.* Philadelphia, J. B. Lippincott Company, 1974, pp. 363-383
10. **Hollinger FB, Werch J, Melnick JL:** A prospective study indicating that double-antibody radioimmunoassay reduces the incidence of post-transfusion hepatitis B. *N Eng J Med 290:1104, 1974*
11. **Villarejos VM, Serra J, Hernandez G:** Endemoepidemic non-parenteral type B hepatitis in Costa Rica. *Am J Med Sci 270:309, 1975*
12. **Dane DS, Cameron CH, Briggs M:** Virus-like particles in serum of patients with Australia antigen-associated hepatitis. *Lancet 1:695, 1970*
13. **Almeda JD, Rubenstein D, Stott EJ:** New antigen-antibody system in Australia antigen-positive hepatitis. *Lancet 2:1225, 1971*
14. **Kaplan PM, Greenman RL, Gerin JL, Purcell WS, Robinson WS:** DNA polymerase associated with human hepatitis B antigen. *J Virol 12:995, 1973*
15. **Robinson WS, Greenman RL:** DNA polymerase in the core of the human hepatitis B virus candidate. *J Virol 13:1231, 1974*
16. **Overby LR, Hung PP, Mao JCH, Ling CM:** Rolling circular DNA associated with Dane particles in hepatitis B virus. *Nature 255:84, 1975*
17. **Magnius LO:** Characterization of a new antigen-antibody system associated with hepatitis B. *Clin Exp Immunol 20:209, 1975*
18. **Hung PP, Mao JCH, Ling CM, Overby LR:** Hybridization of Dane particle DNA with free plasma DNA of hepatitis carriers. *Nature 253:571-572, 1975*
19. **Miller MF:** Principles and Techniques of Electron Microscopy Biological applications, vol. 4. New York, van Nostrand Reinhold Company, 1974, pp. 89-128
20. **Hale EM, Hirata AA, Brusenbach RA, Overby LR:** Antiserum neutralization of bacteriophage Qβ: a mathematical analysis. *J Immunol 102:206, 1969*
21. **Hoofnagle JH, Gerety RJ, Barker LF:** Antibody to hepatitis B virus core in man. *Lancet 2:869, 1973*
22. **Hoofnagle JH, Gerety RJ, Ni LY,** et al.: Antibody to hepatitis B core antigen. A sensitive indicator of hepatitis B virus replication. *N Eng J Med 290:1336, 1974*
23. **Tsuda F, Takahashi T, Takahashi K, et al.:** Determination of antibody to hepatitis B core antigen by means of immune adherence hemagglutination. *J Immunol 115:834, 1975*

RECENT DIAGNOSTIC TECHNIQUES FOR DETECTING HEPATITIS A VIRUS AND ANTIBODY

F. Blaine Hollinger
James E. Maynard

Viral hepatitis is a major health problem in the United States. As one of 30 nationally reported communicable diseases, it currently ranks fourth behind the venereal diseases (gonorrhea and syphilis), varicella and mumps. Under-reporting by physicians — estimated to be 10 to 20 percent of actual cases — has served to underemphasize the considerable economic impact of this disease. In 1972, surveillance reports began including information on HBs Ag testing so as to differentiate reported cases of type B hepatitis from other forms of viral hepatitis. Recent analysis of these data indicates that the etiology of 60 to 80 percent of all reported hepatitis cases is non-B.[1]

In my alloted time, I would like to describe the various diagnostic techniques currently being used by investigators in hepatitis A research and to discuss specific advantages or limitations associated with each. Dr. Dienstag will follow this presentation with a discussion on the epidemiologic characteristics of type A hepatitis infection, using data derived from these same assay systems.

From the Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77030, and the Bureau of Epidemiology, Center for Disease Control, Phoenix, Arizona 85014

F. Blaine Hollinger, M.D., Associate Professor of Virology and Epidemiology, Baylor College of Medicine

James E. Maynard, M.D., Director, Phoenix Laboratories Division, Bureau of Epidemiology, Center for Disease Control

Access to appropriate quantities of specific reagent is fundamental to the development of any method of assay. Since type A hepatitis is transmitted primarily from person to person through the fecal-oral route, the search for virus particles in acute-phase stool specimens has received top priority. In 1973, Feinstone and co-workers,[2] using the technique of immune electron microscopy, reported the visualization of small 27-nm virus-like particles in stool filtrates of experimentally infected hepatitis A volunteers.

IMMUNE ELECTRON MICROSCOPY (IEM)

This is a technique whereby virus particles combine with their specific antibody to form immune aggregates, which are more easily detectable under the electron microscope than are monodispersed virions. In the hands of an experienced investigator, as few as 10^4 to 10^6 particles per ml may be detected, a level which is approximately 1,000 times more sensitive than that described for routine electron microscopy. For IEM detection of hepatitis A virus (HAV)-like particles in stool preparations, 2 percent fecal extracts are prepared by mixing a 0.2-gm aliquot of stool with 10 ml of veal infusion broth containing 0.5 percent bovine serum albumin (BSA).[3] Following the addition of eight to ten 3-mm glass beads, the preparation is shaken manually for 5 min, then clarified at 1500 x *g* for 2 hr at 4°C. The supernatant is removed and filtered

through a series of cellulose acetate membranes (pore sizes, 1.2 μm and 0.45 μm) which have been premoistened with 0.5 percent BSA to reduce virus adsorption.

Detection of HAV-like particles by IEM[4] is achieved by adding 0.1 ml of specific anti-HA serum, diluted 1:10 in phosphate buffered saline, pH 7.4 (PBS: 0.15 M saline containing 0.01 M total phosphate buffer), to 0.9 ml of a 2 percent stool filtrate and incubating the reactants for 1 hr at room temperature. Initial clarification of the anti-HA serum by centrifugation for 1 hr at 60,000 x *g* is recommended to remove any aggregated proteins. Virus-antibody complexes and single particles which are heavily coated with antibody are concentrated by centrifugation at 47,000 x *g* for 90 min at 4°C. After discarding the supernatant, the pellet is resuspended in 2 drops (0.05 ml) of deionized water and rapidly mixed with an equal volume of 3 percent phosphotungstic acid, pH 7.2, for negative staining. A drop of this suspension is placed on a 400-mesh Formvar® carbon-coated copper EM grid. After 1 minute the excess fluid is removed with filter paper and the grid is air-dried. Each grid is examined for typical HAV particles at a magnification of 40,000 to 60,000. If the aggregates are obscured by low molecular weight proteins derived from the serum or the virus preparation, resuspension of the complex in PBS and recentrifugation may improve the EM picture.

For antibody quantification, a fecal extract containing HAV particles is mixed with an unknown serum specimen as previously described. Particles visualized by electron microscopy are scored from 0 (no aggregates) to 4+ (non-glistening aggregates, heavily coated with antibody). Both "full" and "empty" viral capsids have been visualized, some of which appear to exhibit cubic symmetry. Problems associated with this technique include the fact that fecal specimens contain a multitude of bacterial, bacteriophage, viral or other antigens which make differentiation difficult. In addition, antibodies specific for these other agents also may be present in the human antisera used for IEM of HAV.

The hoped-for bonanza of finding prodigious quantities of particles similar to those found in the serum of hepatitis B patients has failed to materialize. Most of the difficulties encountered are related to the fact that initial shedding of particles often precedes the earliest detectable rise in alanine aminotransferase or the onset of prodromal symptoms (Fig. 1). Maximum shedding of virus particles occurs shortly thereafter prior to the onset of jaundice, which is usually the harbinger for cessation of particle shedding. Thus detectable HAV particles are reduced or absent from fecal extracts by the time most patients see their physicians. Large-scale research efforts therefore have been restricted to laboratories fortunate enough to have access to marmosets or chimpanzees or to have stool specimens available from experimently infected volunteers or from large point-source outbreaks.

HEPATITIS A VIRUS PURIFICATION

Despite the cumbersome, time-consuming aspects of IEM, the technique has been essential for identifying those human or subhuman primate stool preparations which are rich in HA virus or antigen (HA Ag). Such preparations form the starting material from which purified HA virus/antigen is obtained for utilization in diagnostic tests. In collaboration with Dr. Dan Bradley and co-workers at the Phoenix Laborator-

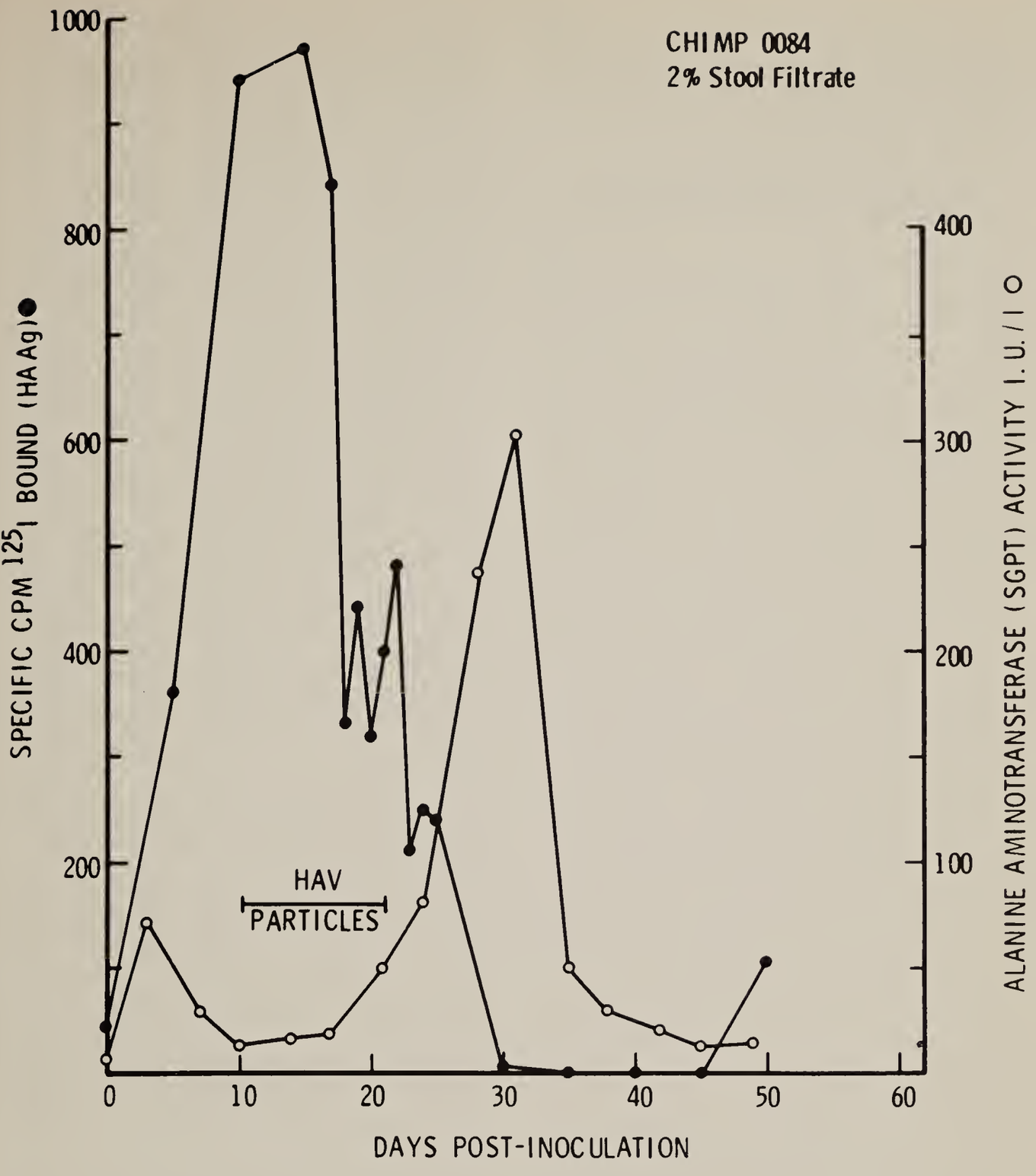


Fig. 1—Detection of HA virus and antigen in serially collected stool specimens from a chimpanzee inoculated intravenously with a 14 to 17 day stool filtrate from another HAV-infected chimpanzee.

ies Division of the Center for Disease Control, a method for purifying HAV is currently being developed.[5,6] Briefly, a fecal extract containing HA virus is concentrated by precipitation with polyethylene glycol. The precipitated proteins are resolubilized and subjected to isopycnic centrifugation in a CsCl gradient. Fractions are assayed for HAV or HA Ag by a microtiter solid-phase immunoradiometric assay (micro-SPIRA, see below) and by IEM. Those fractions containing the highest levels of antigenic reactivity are pooled and chromatographed on Sepharose 2B. Using this method of purification, HAV-like particles are obtained in a relatively pure form suitable for most diagnostic assays. Successful purification of HA Ag also has been

accomplished by isopycnic banding in cesium chloride, rate zonal separation in sucrose, and preparative zonal electrophoresis.[7]

IMMUNE ADHERENCE HEMAGGLUTINATION (IAH)

The limitations of the IEM technique were recognized early, and the need for a rapid, more sensitive assay that could quantitatively evaluate large numbers of specimens became apparent. In 1975, Miller and co-workers,[8] using liver extracts obtained from *S. mystax* marmosets infected with HAV, developed a specific diagnostic complement fixation (CF) test and an immune adherence (IAH) assay for the detection of antibody to HAV (anti-HA). The IAH phenomenon is a specific immunologic reaction in which microorganisms or other antigens complexed with antibody and complement become attached to the surface membrane of untreated primate erythrocytes. The reaction is mediated through the first four components of complement (C1423), although bound C3 is the primary component responsible for mediating the reaction.

The IAH and CF tests have demonstrated good agreement with results of serum neutralization tests performed in marmosets on sera from hepatitis A cases. The CF test is complicated by frequent high levels of anticomplementary activity during the acute phase of the disease which have been attributed to circulating antigen-antibody complexes. Furthermore, low titer CF antibody to normal marmoset liver has been observed frequently.

Preparation of a suitable IAH test antigen has required further purification of the CF liver preparation. This has been accomplished by banding the clarified extract in CsCl, pooling those fractions with a buoyant density of 1.32 to 1.36 g/cc and dialyzing the pooled fractions against PBS. Briefly, the IAH test is performed as follows (Table I):[9] Two-fold dilutions of heat-inactivated serum in a volume of 25 μl are achieved in disposable polyvinyl microtiter "U" plates (#220-24, Cooke Engineering Co., Alexandria, Va.) using veronal buffered saline, pH 7.4, containing 0.1 percent bovine serum albumin (VBD). To each dilution is added 25 μl of viral antigen containing 4 IAH units (previously determined in a two-dimensional antigen: antibody test). The reactants are mixed and incubated for 18 hr at 4°C. Then 25 μl of fresh guinea pig serum diluted 1:75 to 1:100 is added, mixed and the reactants incubated for 40 min at 37°C. To the reactants are added 25μl of a 1 percent human group O erythrocyte suspension (1.2 x 10^8 cells/ml in EDTA-VBD) and 25μl of dithiothreitol (DDT; 3 mg/ml) in 0.04 M EDTA-VBD (2 parts of 0.1 M EDTA, pH 7.5, to 3 parts of VBD). DTT stabilizes C3 in the cell-bound state by protecting it from attack by C3b inactivator. DTT should be prepared fresh since rapid deterioration occurs in less than 1 week at 4 to 8°C.[10] After thorough mixing of the reactants, the plates are incubated for at least 1 hr at room temperature and hemagglutination observed and recorded. Hemagglutination patterns from 3 to 4+ are considered positive (Fig. 2).

Selection of the RBC donor is critical to the optimal performance of the IAH test, since human red blood cells vary in their reactivity, showing degrees of agglutination from marked reactivity (ca. 20 percent of donors) to complete absence of macroscopic hemagglutination (ca. 20 percent of donors).[11,12] The variation does not appear to be solely a function of Rh antigens or Lewis substances, although cells which are *both* Rh-negative and Le(b-) or Le(a+) apparently fail to react or do so minimally in the IAH test.

TABLE I

Scheme for Performing the Immune Adherence Hemagglutination Test

Steps	Incubation Parameters
Prepare two-fold dilutions of heat-inactivated serum in VBD	—
↓ Add 4 IAH units of antigen to each well	Mix, incubate 18 hr at 4°C
↓ Add fresh guinea pig serum	Mix, incubate 40 min at 37°C
↓ Add human group O RBC and dithiothreitol	Mix, incubate 1-3 hr at 25°C
↓ Observe hemagglutination and record	 0 1-2+ 3+ 4+

The IAH test is simple to perform and reportedly is 10 to 100 times more sensitive than the CF test for detecting anti-HA.[8,13] As a method which provides endpoint titers with facility, it represents a welcome addition to our diagnostic armamentarium. However, problems with specificity occasionally do occur and some preparations of HA Ag fail to work satisfactorily. In some cases, the problem can be traced to purity of the antigen or to low concentration. Other factors which may influence the test results include: (i) hemolysis of cells by HA Ag preparations containing the non-ionic detergents NP-40 or Triton X-100; dialysis is beneficial since concentrations below 0.025 percent and 0.01 percent, respectively, fail to cause hemolysis; (ii) presence of CsCl above a buoyant density of 1.07 g/cc (10 percent w/w) inhibits the IAH reaction, presumably by inactivating complement. Dialysis also resolves this problem.

MICROTITER SOLID-PHASE IMMUNORADIOMETRIC ASSAY (MICRO-SPIRA)

The third and final method to be discussed is the highly sensitive and specific micro-SPIRA.[14,14a] Our procedure is a modification of that described by Purcell and co-workers[15,16] and initially was used by us to detect hepatitis B core antigen and antibody in pre-acute and acute phase sera of post-transfusion hepatitis B patients.[17]

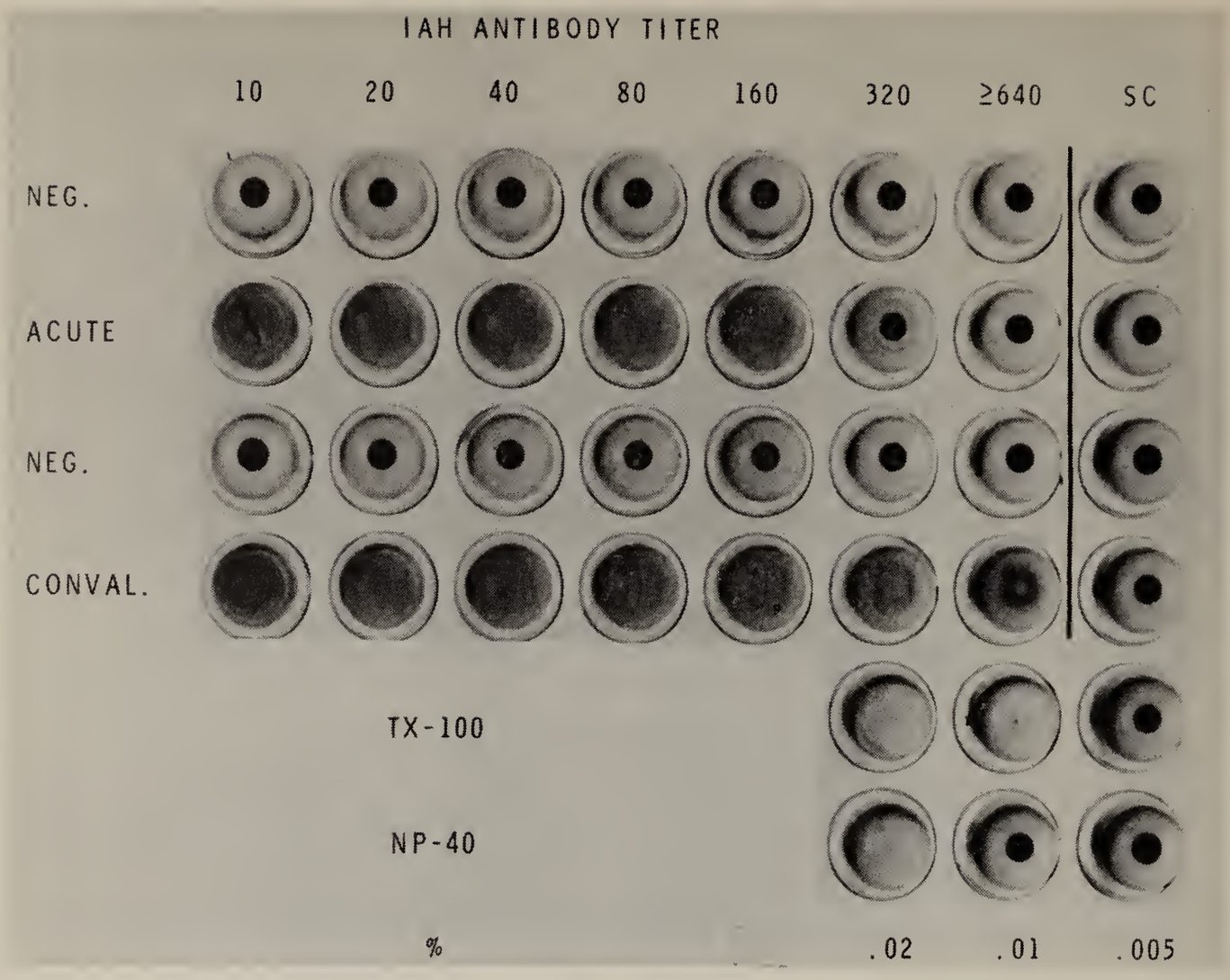


Fig. 2—Detection of antibody in acute and convalescent sera by the immune adherence (IAH) assay. SC = serum (antibody) control. Also shown is the hemolytic effect caused by certain concentrations of the non-ionic detergents NP-40 and Triton X-100. The detergent was added to the test in place of antigen at the percentage (v/v) stipulated.

The micro-SPIRA procedure can be conveniently divided into three stages (Table II): (i) adsorption of unlabeled antibody to the polyvinyl wells of a microtiter plate, (ii) extraction of immunologically active antigen by the coupled antibody, and (iii) detection of bound antigen by combination with labeled specific antibody. It is the direct interaction of antigen with *labeled antibody* rather than competition with *labeled antigen* that distinguishes the two-site immunoradiometric assay from conventional radioimmunoassays. Micro-SPIRA

TABLE II
MAJOR STEPS IN THE MICRO-SPIRA PROCEDURE

Step 1: Adsorption of antibody to microtiter wells
Step 2: Binding of antigen to insolubilized antibody
Step 3: Detection of bound antigen with labeled antibody

$$\text{P/N (positive/negative) ratio} = \frac{\text{cpm of test}}{\text{Mean cpm of negative control samples}}$$

test results are expressed frequently in terms of a P/N (positive/negative) ratio. This is determined by dividing the cpm of the test sample by the mean cpm of the negative control samples. Any alterations in the test conditions which reduce non-specific background counts in the negative control wells and simultaneously stabilize or enhance specific binding will result in a substantial increase in the P/N ratio.

The sensitivity of the micro-SPIRA test can be enhanced by increasing the quantity of antibody adsorbed to the microtiter wells. Recent studies[18] have shown that binding of IgG to polyvinyl surfaces is dependent on (i) the initial concentration of antibody, (ii) time and temperature, and (iii) quality of the adsorbing protein. As shown in Table III, increasing quantities of IgG are adsorbed onto the surface of the plastic over 18 hr at 25°C. Within any given time period, increasing the initial protein concentration increases the total quantity of IgG adsorbed.

Dilution of unlabeled antibody with a protein-containing diluent, such as PBS with BSA, decreases antibody adsorption, especially when immune serum globulin or IgG preparations are used. To illustrate this phenomenon, anti-HA plasma, immune globulin (Ig) and IgG were diluted in PBS containing various concentrations of BSA. After an appropriate incubation period, HA Ag was added to the washed wells, followed 18 hr later by specific ^{125}I-anti-HA IgG. As illustrated in Table IV, dilution of the samples in PBS containing 1 percent BSA (10,000 μg/ml) effectively reduced specific binding of ^{125}I-anti-HA IgG by 12 to 90 percent, presumably by competing favorably with unlabeled antibody for active binding sites on the microtiter wells. Interference gradually diminished until the concentration of BSA reached a level between 1-10 μg/ml, below which no further changes were observed. Cross-comparisons (between plasma and Ig or IgG) should not be extrapolated from these data since P/N ratios are not shown. In general, immune serum globulin preparations are superior to plasma or serum for coating microtiter wells because non-antibody proteins which might interfere with adsorption are removed. Selection of an antiserum of sufficient potency and avidity is essential for detecting low concentrations of HA Ag. This often can be achieved by initially performing a two-dimensional box titration.

TABLE III
ADSORPTION OF LABELED IgG TO POLYVINYL TUBES

Adsorption time (25°C)	Initial protein concentration (μg/ml)	Quantity of IgG adsorbed (μg)
1 hr	100	7.4
	10	4.0
	1	0.7
5 hr	100	7.9
	10	5.8
	1	0.9
18 hr	100	10.2
	10	5.3
	1	1.0

Modified from Herrmann and Collins[18]

TABLE IV
EFFECT OF VARIOUS CONCENTRATIONS OF BOVINE SERUM ALBUMIN (BSA) IN PHOSPHATE BUFFERED SALINE (PBS), pH 7.4, ON INITIAL ANTI-HA ADSORPTION AS MEASURED BY A REDUCTION IN SPECIFIC BINDING OF ^{125}I-ANTI-HA IgG

Concentration of BSA in PBS, pH 7.4 (μg/ml)	Plasma	Anti-HA Ig	IgG
10,000	12%*	51%	90%
1,000	—	39%	84%
100	—	10%	65%
10	—	1%	16%
1	—	—	—
0.1	—	2%	—

*Percentage reduction in specific radioactivity bound to wells.

Fig. 3 schematically illustrates the micro-SPIRA test. For HA Ag detection, 100 μl of an optimal dilution of immune serum globulin or IgG containing anti-HA is added to the wells of a microtiter plate, and adsorption is carried out at room temperature for 18 hours. Sample dessication during incubation is minimized by covering the plate with Parafilm "M" (Dixie/Marathon, Greenwich, Ct.) and a close-fitting plastic lid (#3041, Falcon Plastics, Oxnard, Ca.). The plates are washed four times with 0.15 M saline containing 1:4000 sodium azide, then once with PBS, pH 7.4, containing 2 percent fetal bovine serum (FBS). This protein diluent is employed to remove any weakly adsorbed antibody and is allowed to remain in contact with the wells at room temperature for 15 to 30 minutes to saturate any remaining binding sites. Substitution of 1 percent BSA or 1 percent gelatin for the FBS works equally well, but 1 percent bovine gamma globulin increases background radioactivity significantly.

Following the wash procedure, residual fluid is removed to avoid subsequent dilution of samples or reagent. Specimens suspected of containing HA Ag are added in 50-μl amounts to the wells and incubated at room temperature for 18 hr. The wells are aspirated, washed 5 times with saline, and residual fluid is removed prior to the addition of 75 μl of ^{125}I-anti-HA IgG containing 100,000 to 300,000 cpm. Specific activity of the antibody preparation ranges between 15 and 30 μCi/μg prior to dilution (approximately 1 to 2 atoms of iodine per IgG molecule). Previous studies[14] showed that dilution of labeled IgG in protein-containing diluents, such as PBS with FBS, substantially improve the sensitivity of the micro-SPIRA test. The FBS concentration needed to reduce nonspecific attachment of labeled IgG is quite variable. Thus, each new lot of FBS must be evaluated separately. Optimal concentrations of FBS in PBS have ranged from 12.5 percent to 50 percent in our laboratory. Incubation with the labeled ^{125}I-anti-HA IgG is carried out at 37°C for 4 hr, although 1½ hr at 45°C or 4 hr at room temperature appear to work satisfactorily. Aspiration of the wells, washing and removal of residual fluid are carried out as previously described. Adhesive tape is applied to each plate, and the wells are numbered. Each well is cut out with scissors and placed in a 16 x 125 mm plastic tube for counting in an automatic gamma counter. Several other combinations of incubation times, from 2 hr to 24 hr, and

temperatures, from 4°C to 45°C, have been evaluated. However, in our experience, the parameters described above have proven to be the most sensitive.

For anti-HA detection, the following changes in procedure are required. Unknown samples are diluted 1:10 or 1:20 in PBS containing azide and placed in the first row of a microtiter plate. Three- to sevenfold dilutions can be prepared in PBS with azide by selecting an appropriate microdiluter (25 μl or 50 μl) and by varying the diluent volume (100 μl to 225 μl). Following adsorption for 18 hr at room temperature, the wells are washed, and purified HA Ag is added. The test is completed as described above for the detection of HA Ag. Diminished binding at lower dilutions is frequently observed with potent antisera, which is suggestive of an antigen/antibody prozone. Such a phenomenon is difficult to explain in a solid-phase system and may be due to intermolecular associations of proteins or hydrophilic interactions at those concentrations. In any event, sensitivity of the assay for anti-HA is dependent primarily on the purity and concentration of the HA Ag employed. Appropriate controls are essential for evaluating either the antibody or the antigen test. Their composition is shown in Table V.

Specificity and sensitivity of the micro-SPIRA test for anti-HA has been evaluated under code, using a panel of 21 sera kindly supplied by Dr.

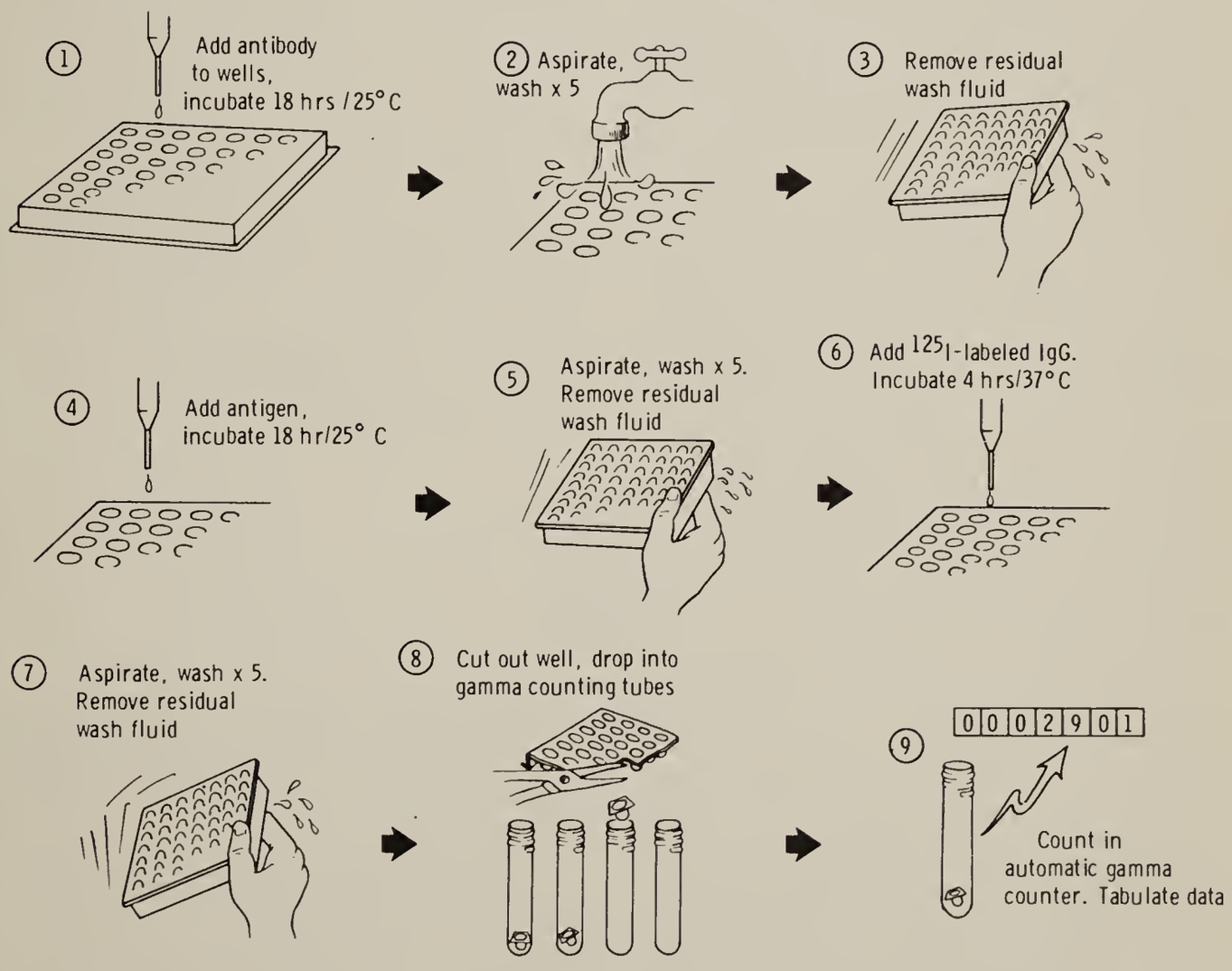


Fig. 3—Schematic illustration of the microtiter solid-phase immunoradiometric assay (micro-SPIRA).

TABLE V
CONTROLS USED IN THE MICRO-SPIRA TEST

CONTROL	Step 1	Step 2	Step 3
ANTIBODY	Antibody or unknown sample	PBS	Label
ANTIGEN	PBS	HA Ag or unknown sample	Label
LABEL	PBS	PBS	Label

J. Dienstag from the National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, Md. This panel consisted of 14 acute and convalescent specimens from naturally or experimentally acquired type A infections and 7 sera from type B hepatitis infections. IAH results were supplied when the code was broken. Good correlation existed between the micro-SPIRA test results and IAH. No false positives were observed. The micro-SPIRA test detected anti-HA in 3 acute hepatitis A specimens which were nonreactive (<1:10) by the IAH test. In two of these, convalescent sera also were supplied and seroconversion occurred. Although anti-HA was observed in sera from 4 of 7 type B hepatitis cases, no significant changes in titer occurred between the acute and convalescent specimens. Precision (agreement between duplicate samples tested under code) was excellent. In general, micro-SPIRA antibody titers were at least 100-fold greater than IAH titers.

Purcell and co-workers[19] use a blocking test to measure anti-HA. Briefly, microtiter wells coated with anti-HA are incubated with HA Ag, then washed, and the residual fluid removed. Decimal dilutions of the sera to be tested are added and incubation continued overnight at 4°C. Following an additional washing step, labeled anti-HA is added and the test resumed similar to that described above. Reduction in radioactivity of 40 percent or more when compared to an anti-HA negative serum is considered evidence for the presence of anti-HA in the sample.

The micro-SPIRA technique has shown great potential and appears to be more sensitive than IAH for detecting HA Ag and anti-HA. Scarcity of purified antigen remains the only limiting factor delaying widespread application of these techniques.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Jules Dienstag, NIH, NIAID, for kindly supplying the anti-HA serum panel. The technical expertise of Susan Fogg, Cathy Morton, and Cecil Hornbeck is gratefully acknowledged.

REFERENCES

1. **Bryan JA, Gregg MB:** Viral hepatitis in the United States 1970-1973: an analysis of morbidity trends and the impact of HBsAg testing on surveillance and epidemiology. *Amer J Med Sci 270:271, 1975*

2. **Feinstone SM, Kapikian AZ, Purcell RH:** Hepatitis A: detection by immune electron microscopy of a virus-like antigen associated with acute illness. *Science 182:1026, 1973*

3. Research Reference Reagents Note #17, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md

4. **Kapikian AZ, Wyatt RG, Dolin R, Thornhill**

TS, Kalica AR, Chanock RM: Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J Virol 10:1075, 1972*

5. **Bradley DW, Hollinger FB, Hornbeck CL, Maynard JE:** Isolation and characterization of hepatitis A virus. *Amer J Clin Pathol 65:876, 1976*

6. **Hornbeck CL, Gravelle CR, Hollinger FB, Bradley DW:** Agarose gel filtration of concentrated fecal extracts containing virus-like particles associated with hepatitis A. *Intervirology (in press)*

7. **Moritsugu Y, Dienstag JL, Valdesuso J, Wong DC, Wagner J, Routenberg JA, Purcell RH:** Purification of HA Ag from feces and detection of antigen and antibody by immune adherence hemagglutination. *Infect Immun 13:898, 1976*

8. **Miller WJ, Provost PJ, McAleer WJ, Ittensohn OL, Villarejos VM, Hilleman MR:** Specific immune adherence assay for human hepatitis A antibody. Application to diagnostic and epidemiologic investigations. *Proc Soc Exp Biol Med 149:254, 1975*

9. **Mayumi M, Okochi K, Nishioka K:** Detection of Australia antigen by means of immune adherence haemagglutination test. *Vox Sang 20:178, 1971*

10. **Olson PR, Welbien BJ, O'Leary JJ, Moscowitz AJ, McCullough J:** A simple technique for the inactivation of IgM antibodies using dithiothreitol. *Vox Sang 30:149, 1976*

11. **Gozin D, Souller JP:** Attempt to select human group O red cells for use in the immunoadherence test for detection of the Australia antigen. *Vox Sang 23:472, 1972*

12. **Schwartz J, Levy R, Vardinon N:** Human red cell receptors and immune adherence haemagglutination. *Experientia 28:1097, 1972*

13. **Krugman S, Ward R, Giles JP, et al.:** Infectious hepatitis: detection of virus during the incubation period and in clinically inapparent infection. *N Engl J Med 261:729, 1959*

14. **Hollinger FB, Bradley DW, Maynard JE, Dreesman GR, Melnick JL:** Detection of Hepatitis: A viral antigen by radioimmunoassay. *J Immunol 115:1464, 1975*

14a. **Hollinger FB, Bradley DW, Dreesman GR, Melnick JL:** Detection of viral hepatitis type A. *Amer J Clin Pathol 65:854, 1976*

15. **Purcell RH, Gerin JL, Almeida JB, Holland PV:** Radioimmunoassay for the detection of the core of the Dane particle and antibody to it. *Intervirology 2:231, 1973/74*

16. **Purcell RH, Wong DC, Alter HJ, Holland PV:** Microtiter solid-phase radioimmunoassay for hepatitis B antigen. *Appl Microbiol 26:478, 1973*

17. **Hollinger FB, Dreesman GR, Fields H, Melnick JL:** Hbc Ag, anti-HBc, and DNA polymerase activity in transfused recipients followed prospectively. *Am J Med Sci 270:343, 1975*

18. **Herrmann JE, Collins MF:** Quantitation of immunoglobulin adsorption to plastics. *J Immunol Methods, 10:363, 1976*

19. **Purcell RH, Wong DC, Moritsugu Y, Dienstag JL, Routenberg JA, Boggs JD:** A microtiter solid-phase radioimmunoassay for hepatitis A antigen and antibody. *J Immunol 116:349, 1976*

VIRAL HEPATITIS, TYPE A: ETIOLOGY AND EPIDEMIOLOGY

Jules L. Dienstag

Robert H. Purcell

ABSTRACT. Studies in man and nonhuman primates during the previous three decades provided a groundwork for the current acceleration in our understanding of viral hepatitis, type A. Recently, a 27 nm virus-like particle, designated hepatitis A antigen (HA Ag), was detected in stools of patients with acute type A viral hepatitis and, subsequently, in the sera and livers of infected marmosets. The discovery of HA Ag was instrumental in the development of several *in vitro* diagnostic tests to identify both antigen and antibody. With these new techniques, we and others have been able to characterize and purify HA Ag particles, demonstrate the transmission of hepatitis A virus in chimpanzees, test the susceptibility of other nonhuman primates, and study the immunology and seroepidemiology of hepatitis A virus infection. Evidence accumulating from these studies suggests that HA Ag is the etiologic agent of type A viral hepatitis. In addition, seroepidemiologic investigations with these new diagnostic techniques have identified a new category of viral hepatitis unrelated to either hepatitis A or B viruses, so called "non-A, non-B" hepatitis.

Blumberg's discovery of an antigen (Australia antigen or hepatitis B surface antigen) associated with viral hepatitis, type B[1] preceded by almost a decade the discovery of an immunologic marker for type A viral hepatitis.[2] During the interval between these discoveries, most investigators concentrated on the characterization of type B hepatitis, leading to rapid progress in our understanding and control of viral hepatitis, type B, as described earlier in this symposium. In the absence of a serologic marker for hepatitis A virus (HAV), progress was limited in our understanding of viral hepatitis, type A.

Descriptions of the clinical entity now associated with HAV infection have appeared since antiquity; however, its characterization as a viral illness of short incubation (15 to 50 days), transmitted primarily by close contact via the fecal-oral route was not established until the first half of the twentieth century.[3-11] Overlapping epidemiologic and clinical distinctions between this form of "infectious hepatitis" and a disease of longer incubation commonly associated with parenteral inoculation ("serum hepatitis") prompted MacCallum in 1947 to designate them as hepatitis A and hepatitis

From the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 40014

Jules L. Dienstag, M.D., formerly: Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH; now Gastrointestinal Unit, Massachusetts General Hospital, Boston, Mass. 02114

Robert H. Purcell, M.D., Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH

B, respectively.[12]

Despite numerous claims of success, no report of HAV cultivation *in vitro* has ever been confirmed. Consequently, interested investigators were attracted to other experimental approaches. Two lineages of study emerged during the previous three decades and provided the groundwork for the current acceleration in our understanding of type A viral hepatitis. The first of these, undertaken before attempts to transmit HAV to animals were successful, were studies performed in human volunteers, often among military populations. From the earliest of these investigations emerged the understanding that HAV was a small, ether-and-heat-resistant virus; it could be detected in the feces and blood of infected patients during the incubation period and acute illness; and it was immunologically distinct from hepatitis B virus, eliciting homologous but not heterologous immunity.[5,7-11,13,14]

Of these volunteer studies, two assume special importance. In the 1950's and 1960's Krugman and his colleagues studied the epidemiology and clinical course of viral hepatitis among children at the Willowbrook State School, an institution in which hepatitis is endemic.[15] During the course of these studies Krugman distinguished the endemic MS-1 strain which we know today is an HAV strain, from MS-2, a hepatitis B virus strain, and demonstrated the infectivity of serum and feces of patients with type A hepatitis.[16] In addition, serial collections of feces and serum were stored and made available to subsequent investigators. Descending directly from the Willowbrook studies, the second and last major volunteer study of importance to hepatitis A research was conducted in 1968 among volunteers at the State Prison in Joliet, Illinois.[17] MS-1 serum provided by Krugman was transmitted to and serially passaged in adults. Large quantities of infectious material—stool, serum, and plasma—were collected for attempts to cultivate and identify HAV. Both the Willowbrook and Joliet studies became pivotal for subsequent work.

The second major area of investigation basic to current advances was the development of an experimental animal model for HAV infection. In 1965, Deinhardt, Holmes, Capps, and Popper reported the first convincing and reproducible successful transmission of human hepatitis to several species *(Saguinus fuscicollis, S. nigricollis,* and *S. oedipus)* of marmosets (tamarins), small South American monkeys.[18,19] Although the initially passaged and extensively studied GB agent was eventually shown not to be HAV,[20] in subsequent studies performed under code, Deinhardt and his colleagues produced hepatitis in marmosets inoculated with acute phase serum from MS-1-infected Joliet volunteers.[21] Susceptibility to human HAV was even greater among *Saguinus mystax* marmosets, a species first proposed by Hillis,[22] utilized by Lorenz *et al.*,[23] and evaluated extensively by Mascoli, Provost, and colleagues at Merck Sharp and Dohme.[24,25] The latter group transmitted hepatitis to marmosets by inoculation with serum from Costa Rican patients with naturally-acquired type A viral hepatitis. The CR326 strain was studied extensively and used to develop an assay for neutralizing antibody to HAV.[25] This same strain played an important role in more recent advances to be described below. A test for neutralizing antibody was described by Holmes *et al.*[20] at the same time. Though of limited practical applicability, the development of an assay for neutralizing antibody was the first test to identify hepatitis A virus infection in other than human hosts.

Visualization of hepatitis A antigen

A new era began for hepatitis A research when Feinstone *et al.* reported the detection by immune electron microscopy (IEM) of 27 nm virus-like antigen particles (Fig. 1) in acute phase stools of Joliet prison volunteers *(vide supra)* inoculated with the MS-1 strain of HAV.[2] These particles, designated hepatitis A antigen (HA Ag), were visualized in aggregates or as single particles coated with antibody, when acute phase stools were incubated with convalescent serum. Particles were not detected when pre-inoculation or convalescent stools were used or when incubation was with pre-inoculation serum from patients with type A viral hepatitis. Once identified, stool filtrates containing HA Ag were used as a reagent to detect antibody to HA Ag (anti-HA), and the specificity of the IEM assay was confirmed by demonstrating a serologic response in paired sera from patients with experimental or naturally-occurring HAV infection but not from patients with type B viral hepatitis or viral gastroenteritis.

The impact of earlier experimental marmoset infection studies became even more profound when, shortly after Feinstone's report describing HA Ag particles in human stools, Provost *et al.*[26] reported the detection of morphologically similar HA Ag particles in the sera and livers of *S. mystax* marmosets experimentally infected with the CR326 strain of human HAV. The almost simultaneous discovery in different laboratories of HA Ag from different sources and the confirmation of these findings in other laboratories,[27,28] was instrumental in the development of IEM,[2] complement fixation (CF),[29,30] immune adherence hemagglutination (IAHA),[30-32] and solid phase radioimmunoassay (SPRIA)[33,34] diagnostic tests to identify both antigen and antibody. (Details of these *in vitro* assays appear earlier in this symposium.) This armamentarium of sensitive tests specific for HAV infection has provided the key to rapid progress in our understanding of type A hepatitis.

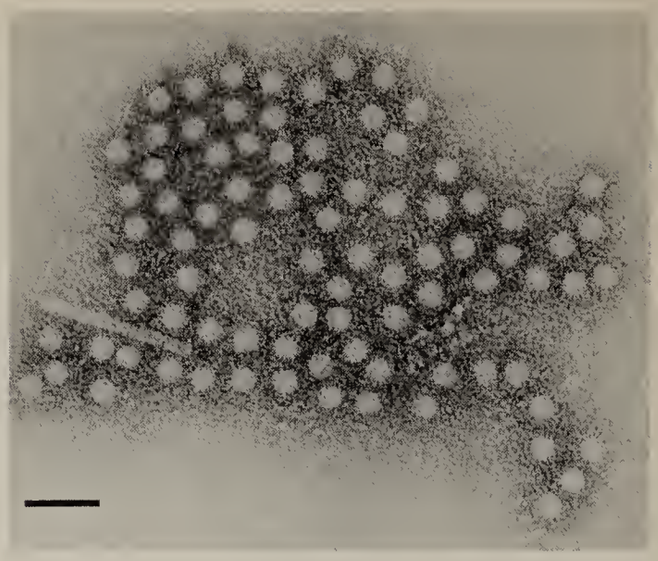


Fig. 1—An aggregate of human stool-derived HA Ag particles coated with antibody. The bar represents 100 nm. (2 percent phosphotungstic acid negative stain).

Fecal HA Ag shedding pattern

In an attempt to establish further the etiologic role of HA Ag particles in type A hepatitis and to determine the temporal relationship of fecal shedding of HA Ag particles to clinical hapatitis, we tested serial stool specimens from Joliet Prison volunteers for HA Ag particles by IEM.[35] The appearance of HA Ag particles in stools coincided with the development of nonspecific symptoms, such as malaise, fatigue, fever, abdominal pain, and loss of appetite, but particle shedding reached a peak, declined, and ceased just before jaundice became clinically and biochemically apparent. Elevation of serum amino-

transferase activity and morphologic changes in the liver consistent with acute hepatitis followed HA Ag particle excretion, which occurs early during the course of illness and rarely persists when a patient presents with clinically overt hepatitis. Given the sensitivity threshold of IEM, the pattern of early fecal HA Ag shedding detected with this *in vitro* method correlated well with the early infectivity of feces noted by Krugman[15] and further supported an etiologic role for HA Ag particles in type A viral hepatitis.

Natural outbreaks of viral hepatitis, type A

Confirmation of the detection of HA Ag particles came from evaluation of natural outbreaks of viral hepatitis epidemiologically compatible with type A disease. After the initial detection of HA Ag, published or preliminary reports appeared describing natural outbreaks in Arizona,[28] Australia,[27] California,[36] Alaska (Maynard, J.E., personal communication), Germany (Deinhardt, F., Cross, G., personal communication), Argentina (Fay, O.H., personal communication), and Costa Rica (Provost, P.J., personal communication). In each instance, HA Ag was detected in early acute phase stool samples of affected patients. When shedding patterns of HA Ag in stools were studied, the early pattern described above for experimental illness was confirmed. Selective exchange of reagents among investigators revealed that the HA Ag particles isolated in each outbreak were immunologically indistinguishable from the others or from the original Joliet stool (MS-1 derived) HA Ag particles. Similarly, serologic responses to HA Ag were demonstrated in serum pairs from patients in each of these outbreaks as well as from individuals with naturally-occurring type A hepatitis in the South Pacific, North and South America, suggesting that strains of HAV from all over the world are antigenically similar, if not identical. In the event future studies bear out these findings, development of a univalent vaccine, effective worldwide, will be simplified.

Besides the importance of natural outbreaks for their epidemiologic significance, human stools collected during these recent outbreaks have provided a rich source of antigen for use in the new serologic tests. Most notably, stool-derived HA Ag, after appropriate purification, compares favorably with marmoset liver-derived HA Ag as a source of antigen for IAHA.[32] The present depletion of marmoset supplies for medical research makes these human stools an even more important research resource.

Experimental infection of chimpanzees

For many years, attempts to transmit human HAV infection to chimpanzees had been disappointing or inconclusive;[37] however, interest in chimpanzees as a susceptible host for HAV was maintained by reports of short-incubation hepatitis among handlers of newly imported chimpanzees[38] and by the demonstration of the susceptibility of chimpanzees to human hepatitis B virus.[39] Earlier failures to infect chimpanzees with HAV may have resulted from the use of poorly defined inocula and inclusion in those studies of animals that had developed immunity during a previous exposure. Availability of new assays to detect HA Ag and anti-HA provided the nec-

essary immunologic markers to reassess the susceptibility of chimpanzees to HAV infection. When seronegative chimpanzees were identified and inoculated, orally or parenterally, with stool filtrates containing HA Ag particles, investigators in several laboratories demonstrated the development of clinical, biochemical, and morphologic evidence of acute hepatitis in these nonhuman primate hosts. [40-42] The typical course of events paralleled that seen in man: (a) fecal HA Ag shedding was detected, by IEM or SPRIA, as early as two weeks after inoculation, preceding elevation of aminotransferase activity and ending when peak SGPT levels had been reached; (b) anti-HA was detectable by IEM during acute liver injury, suggesting a pathophysiologic role for the humoral immune response in type A hepatitis, and by IAHA three to four weeks later; and (c) histologic changes occurred in the liver, namely: focal necrosis of hepatocytes with replacement by macrophages and lymphocytes; a mixed mononuclear portal inflammatory infiltrate; and the appearance of acidophilic bodies, all compatible with a mild acute viral hepatitis that resolved with no significant sequelae.

In addition, HA Ag particles have been detected in chimpanzee liver homogenates by IEM, as well as 27 nm virus-like structures in cytoplasmic vesicles by thin-section electron microscopy[43] confirming similar findings in HAV-infected marmosets.[26] Furthermore, the recent demonstration of HA Ag particles in bile of HAV-infected chimpanzees[43] lends support to the concept that HAV replicates in the liver and is released via biliary excretion into the gut where it gains access to feces. The infectivity, in chimpanzees and in marmosets, of stool filtrates containing HA Ag lends additional support to the concept that HA Ag is the hepatitis A virus.

Susceptibility of other nonhuman primates to hepatitis A virus

In collaboration with Dr. William T. London, we have tested the susceptibility to HAV infection of nonhuman primates other than marmosets and chimpanzees. Evidence of exposure to HAV was detected in 13 percent to 60 percent of cynomolgus monkeys, owl monkeys, rhesus monkeys, patas monkeys, pigtail monkeys, spider monkeys, and stumptail monkeys. Anti-HA has been detected also in grivets by Miller *et al.*[31] In addition, anti-HA seroconversions have been detected among African green monkeys, baboons, cebus monkeys, rhesus monkeys, owl monkeys and woolly monkeys injected with HA Ag-containing inocula previously shown to be infectious in chimpanzees and marmosets; however, no elevation of aminotransferase activity or liver morphologic changes have been observed in any of these animals. Whether the observed seroconversions represent true infection or immunization without infection is not yet clear, but, no nonhuman primate tested has proven as susceptible as marmosets and chimpanzees.

Characterization of HA Ag

Morphologically, HA Ag particles are 27 nm in diameter and appear to have cubic symmetry. Whether derived from human stool (Fig. 1),[2] marmoset liver (Fig. 2), serum[26] or stool,[44] or chimpanzee liver, bile (Fig. 3), or stool,[43] all HA Ag particles are morphologically indistinguishable, appearing either penetrated or non-penetrated by negative stain. Distinct from other antigen particles found in stools[45] HA Ag particles from all sources are immunologically similar, reacting with antibody in convalescent but

not preinoculation serum from patients with HAV infection. Moreover, antisera raised in guinea pigs against partially purified marmoset-liver-derived or human-stool-derived, HA Ag were immunologically cross-reactive,[45] providing further evidence to support the similarity of HA Ag from different sources.

On the other hand, a multiplicity of buoyant densities in cesium chloride (CsCl) has been reported for HA Ag particles. Hepatitis A antigen particles in stool have been found to band at a high density, with peaks ranging from 1.38 g/cm^3 to 1.40 g/cm^3 [27,32,41,43,46,47] and at a lower density, with a range of peaks from 1.31 g/cm^3 to 1.34 g/cm^3. [32,44,47,48] Provost *et al.*[26] reported a buoyant density of 1.34 g/cm^3 for HA Ag particles detected in marmoset liver and serum, and Schulman *et al.*[43] confirmed this density for marmoset and chimpanzee liver-derived HA Ag particles. Hollinger *et al.*,[48] however, detected a major peak of 1.27 g/cm^3 for particles derived from marmoset and chimpanzee liver. No morphologic or immunologic distinctions have been noted for particles at different densities, and infectivity resides in fractions of both high and low density particles. In bile, however, Schulman *et al.*[43] detected a population of particles not penetrated by negative strain ("full") at a density of 1.34 g/cm^3 and another population penetrated by negative stain ("empty") at a density of 1.29 g/cm^3. An additional distinction has been noted between the relative instability of 1.4 g/cm^3 particles and the stability of the 1.34 g/cm^3 population in stool.[32]

Despite the heterogeneity of HA Ag banding profiles, Provost *et al.*[26] have suggested that, based on a predominant density of 1.34 g/cm^3, cytoplasmic localization in hepatocytes, and preliminary suggestive acridine orange staining, HAV is an RNA virus probably of the entero-

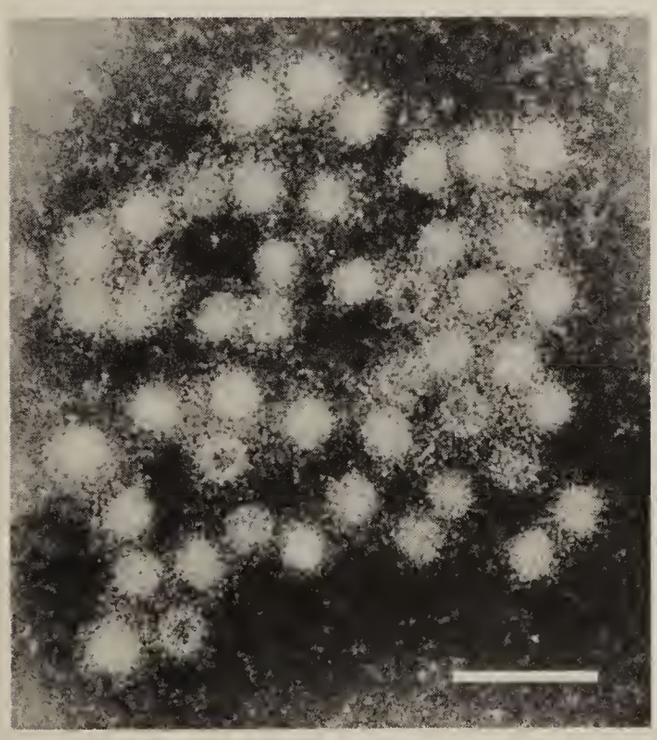


Fig. 2—An aggregate of *S. mystax* marmoset liver-derived HA Ag particles heavily coated with antibody. The bar represents 100 nm. (2 percent phosphotungstic acid negative stain).

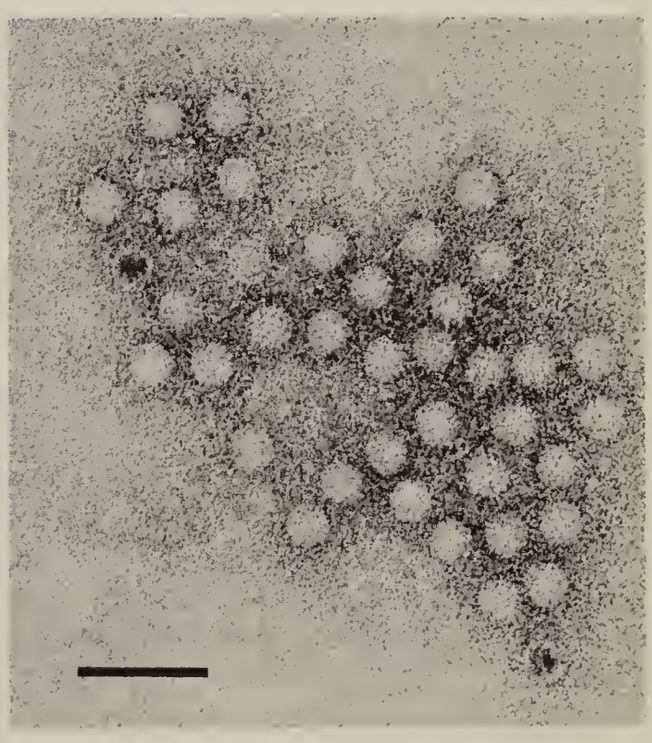


Fig. 3—An aggregate of chimpanzee bile-derived HA Ag particles coated with antibody. The bar represents 100 nm. (2 percent phosphotungstic acid negative stain).

virus class. On the other hand, although high density (>1.4 g/cm^3) enteroviruses have been observed,[49] as a rule it is uncommon for enteroviruses to have multiple density populations, and the heat-stability of HAV is distinctly uncommon among enteroviruses. Before HAV can be classified definitively, additional purification and characterization will be required. What physical properties of HA Ag particles have been recorded have been utilized to purify HA Ag from liver and feces, as described in a previous report in this symposium.

Immune response to hepatitis A antigen

Quantitative techniques to identify anti-HA have been applied to the study of antibody kinetics during and after HAV infection. Antibody detected by IEM or CF can be detected during acute illness in man, as noted above for chimpanzees, whereas the appearance of anti-HA detected by IAHA is delayed from one to four weeks.[30,32] Subsequently IAHA antibody levels rise gradually towards a peak two to three months after acute illness,[30,31] occasionally reaching titers as high as 1:300,000 and proceed to fall thereafter; however, even five to 10 years after illness, relatively high serum anti-HA titers are maintained.[30,31]

Infection with HAV confers homologous immunity against reinfection, as deduced by Havens from studies in volunteers[13] and Krugman from studies at Willowbrook.[16] Studies in which marmosets and chimpanzees have been rechallenged with HA Ag-containing inocula after a primary infection confirm this conclusion. No abnormalities of liver function or histology can be detected after the secondary inoculation; however, an antibody response characteristic of hyperimmunization, an immediate, transient boost in titer, occurs and can be measured.

In subclinical cases identified by elevations of aminotransferase activity alone, anti-HA seroconversions have been detected, confirming HAV illness[36] (Villarejos, V.M. personal communication). Even more significantly, however, immune responses typical of that seen in clinically apparent infection have now been detected in the absence of liver function abnormalities in individuals previously felt to have escaped infection.[50] Reappraisal of the results of earlier studies with newly available serologic techniques may change some of our concepts about type A hepatitis, its epidemiology, and its immunoprophylaxis.

Seroepidemiology of type A hepatitis

Age and class-related exposure to HAV: Epidemiologic observations in the past have suggested that HAV depends for its spread upon poor personal hygiene, suboptimal sanitation, and crowded living conditions among its human hosts. Limited serologic surveys of age-related and socioeconomic class-related frequency of anti-HA confirm these epidemiologically-derived conclusions. Purcell *et al.*[45] demonstrated a direct relationship between frequency of antibody and increasing age; sera of randomly selected individuals under 20 years of age contained no anti-HA, whereas greater than 80 percent of individuals tested above the age of 40 had serologic evidence of exposure to HAV. The fact that children and young adults harbored no antibody to a virus traditionally thought to infect this age group suggests that recent improvements in hygiene and public health have changed the epidemiology of type A hepatitis in children, paralleling the experience with poliovirus.

In a comparison of individuals of different socioeconomic classes, Miller *et al.*[31] found that 14 percent of 22 middle class individuals, 33 percent of 33 commercial blood donors, generally of low socioeconomic background, and 62 percent of 26 prisoners had serum anti-HA. Thus, exposure seems to be inversely related to increasing socioeconomic status, indicating that susceptibility extends into early adulthood for individuals whose means and living conditions are more amenable to adequate hygiene.

Transfusion-associated hepatitis: When the association between HB_sAg and type B hepatitis was discovered and screening of blood donors implemented, and when the high frequency of hepatitis among transfused patients receiving commercial blood was noted and commercial blood donation discouraged, the elimination of posttransfusion hepatitis was anticipated. It became apparent, however, that these measures fell short of eradicating the transfusion-associated hepatitis problem. Because some cases of post-transfusion hepatitis have a short incubation period and because type A hepatitis can be transmitted by parenteral inoculation, it was reasonable to conclude that HAV is responsible for the residual cases of non-B transfusion-related hepatitis. Following the development of assays to detect anti-HA, paired sera from HB_sAg-negative post-transfusion hepatitis patients were evaluated for a serologic response indicative of HAV infection; however, none of more than 50 cases of hepatitis evaluated in three studies[51-53] could be related serologically to HAV. Thus, although HAV is transmissible parenterally, its short incubation period, its even shorter viremia, and the apparent absence of chronic HA Ag carriers make HAV an unlikely cause of transfusion-associated hepatitis.

Modes of transmission of HAV: In addition to its role in hepatitis related to blood transfusion, the role of HAV in other modes of hepatitis transmission is being re-examined with the new serologic techniques. In each of the epidemiologic categories of transmission classically associated with type A hepatitis, a serologic relationship to Krugman's MS-1 strain of HAV has been demonstrated. The etiologic role of HAV has been serologically confirmed in several common sources of water-borne[2] and food-borne outbreaks,[28,36] including an outbreak related to ingestion of contaminated shellfish.[54] Several intra-family outbreaks and intra-institutional outbreaks have been evaluated and related serologically to HAV infection.[2,45] In addition, preliminary evidence from several laboratories indicates that HAV is responsible for cases of hepatitis among chimpanzee handlers.[55] And for a significant proportion of sporadic hepatitis with no obvious epidemiologic source (Gust, I.D., Mosely, J. W., Morissugu, Y., unpublished data).

Evidence for new hepatitis viruses; As mentioned above, cases of post-transfusion hepatitis unrelated to hepatitis B virus had been assumed to result from HAV infection: however, a reappraisal of incubation periods[56] and the application of serologic tests for HAV infection[51-53] demonstrated that these cases were unrelated to both hepatitis A and B viruses. Neither could cytomegalovirus or Epstein-Barr virus be implicated serologically in a significant proportion of these cases. The fact that elimination of commercial blood donors reduces the in-

cidence of both type B and non-B hepatitis adds even more weight to the argument for the existence of other hepatitis viruses. Moreover, serologic evaluation of other epidemiologic modes of hepatitis has unearthed cases of "non-A, non-B" hepatitis in an intra-family outbreak,[57] among institutionalized individuals (Ogra, P., unpublished data), and among patients presenting with acute hepatitis in a non-epidemic setting (sporadic hepatitis). Suggestive evidence exists, too, for the etiologic roles of non-A, non-B virus(es) in renal transplant (Combes, B., private communication) and hemodialysis-associated hepatitis.[58] The potential existence of one or more new hepatitis viruses ranks among the most exciting new developments to evolve from the application of serologic assays for HAV infection, and the search for these agents is attracting many investigators. Ironically, the surgeon from whom the GB agent of Deinhardt *et al.*[19] was isolated, apparently had a case of non-A, non-B hepatitis, as determined by recent serologic testing. As Deinhardt has suggested, the GB agent may be a "non-A, non-B" or "type C" virus;[44] however, presently no serological tool is available for its detection in patients with hepatitis.

Immunoprophylaxis

The ultimate goal of hepatitis research is to control or eliminate viral hepatitis by immunization, ideally with an inactivated or attenuated live vaccine. Active immunization of this kind, however, probably must await the cultivation of HAV in tissue culture. In the absence of this alternative, passive immunization with immune serum globulin is routine practice for contacts of patients with type A hepatitis. The serologic means are available for the first time to identify globulin lots with high titer anti-HA and to avoid inoculating individuals already immune. A pilot survey of 24 commercial globulin lots was conducted by Miller *et al.*[31] who found anti-HA titers ranging from 1:1000 to 1:16,000. Thus, although earlier studies showed that immune serum globulin modifies rather than prevents illness, conceivably, protection varies as a function of anti-HA titer; the protective effect of high titer globulin can now be re-evaluated in prospective studies.

CONCLUSIONS

Evidence accumulating from these studies suggests that HA Ag is the etiologic agent of hepatitis A. The discovery of this viral antigen and serologic techniques to detect antibody to it have accelerated progress in our understanding of type A hepatitis, have led to the development of a new animal model, have allowed re-evaluation of epidemiological concepts about type A hepatitis, and have suggested the existence of other hepatitis viruses.

ACKNOWLEDGMENTS

We thank Mrs. Virginia Frye for preparation of the manuscript, Miss Dinah Bertran for photographic assistance, and the numerous investigators with whom we have collaborated in the studies cited.

REFERENCES

1. **Blumberg BS, Alter HJ, Visnich S:** A "new" antigen in leukemia sera. *JAMA. 191:541, 1965*

2. **Feinstone SM, Kapikian AZ, Purcell RH:** Hepatitis A: Detection by immune electron microscopy of a virus-like antigen associated with acute illness. *Science 182:1026, 1973*

3. **Williams H:** Epidemic jaundice in New York State 1921-1922. *JAMA 80:532, 1923*

4. **Blumer GB:** Infectious jaundice in the United States. *JAMA 81:353, 1923*

5. **Voegt H:** Zur aetiologie der hepatitis epidemica. *Munchener Med Wochenschr 89:76, 1942*

6. **Cameron JDS:** Infective hepatitis. *Quart J Med 12:139, 1943*

7. **MacCallum FO, Bradley WH:** Transmission of infective hepatitis to human volunteers. *Lancet 2:228, 1944*

8. **Findlay GM, Wilcox RR:** Transmission of infective hepatitis by faeces and urine. *Lancet 1:212, 1945*

9. **Paul JR Jr, Havens WP Jr, Sabin AB, Philip CB:** Transmission experiments in serum jaundice and infectious hepatitis. *JAMA 128:911, 1945*

10. **Neefe JR, Gellis SS, Stokes J Jr:** Homologous serum hepatitis and infectious (epidemic) hepatitis: Studies in volunteers bearing on immunological and other characteristics of the etiological agents. *Am J Med 1:3, 1946*

11. **Havens WP Jr:** Period of infectivity of patients with experimentally induced infectious hepatitis. *J Exp Med 83:251, 1946*

12. **MacCallum FO:** Paper presented to the International Conference of Physicians. *Lancet 2:435, 1947*

13. **Havens WP Jr:** Experiment in cross immunity between infectious hepatitis and homologous serum jaundice. *Proc Soc Exp Biol Med 59:148, 1945*

14. **Havens WP Jr:** Properties of the etiologic agent of infectious hepatitis. *Proc Soc Exp Biol Med 58:203, 1945*

15. **Krugman S, Ward R, Giles JP:** The natural history of infectious hepatitis. *Am J Med 32:717, 1962*

16. **Krugman S, Giles JP, Hammond J:** Infectious hepatitis: Evidence for two distinctive clinical, epidemiological, and immunological types of infection. *JAMA 200:365, 1967*

17. **Boggs JD, Melnick JL, Conrad ME, Felsher BF:** Viral hepatitis: Clinical and tissue culture studies. *JAMA 214:1041, 1970*

18. **Deinhardt F, Holmes AW:** Epidemiology and etiology of viral hepatitis. *In "Progress in Liver Diseases" Vol II,* Popper H, Schaffner F, editors, New York, Grune and Stratton, 1965, pp. 373-394

19. **Deinhardt F, Holmes AW, Capps RB, Popper H:** Studies on the transmission of human viral hepatitis to marmoset monkeys. I. Transmission of disease, serial passages and description of liver lesions. *J Exp Med 125:673, 1967*

20. **Holmes AW, Deinhardt F, Wolfe L, Froesner G, Peterson D, Casto B:** Specific neutralization of human hepatitis type A in marmoset monkeys. *Nature 243:419, 1973*

21. **Holmes AW, Wolfe L, Rosenblate H, Deinhardt F:** Hepatitis in marmosets: Induction of disease with coded specimens from a human volunteer study. *Science 165:816, 1969*

22. **Hillis WD,** Viral hepatitis: An unconquered foe. *Military Med 133: 343, 1968*

23. **Lorenz D, Barker L, Stevens D, Peterson M, Kirschstein R:** Hepatitis in the marmoset, *Saguinus mystax. Proc Soc Exp Biol Med 135:348, 1970*

24. **Mascoli CC, Ittensohn OL, Villarejos VM, Arguedas JA, Provost PJ, Hilleman MR:** Recovery of hepatitis agents in the marmoset from human cases occurring in Costa Rica. *Proc Soc Exp Biol Med 142:276, 1973*

25. **Provost PJ, Ittensohn OL, Villarejos VM, Arguedas JA, Hilleman MR:** Etiologic relationship of marmoset-propagated CR326 hepatitis A virus to hepatitis in man. *Proc Soc Exp Biol Med 142:1257, 1973*

26. **Provost PJ, Wolanski BS, Miller WJ, Ittensohn OL, McAleer WJ, Hilleman MR:** Physical, chemical and morphologic dimensions of human hepatitis A virus strain CR326. *Proc Soc Exp Biol Med 148:532, 1975*

27. **Locarnini SA, Ferris AA, Stott AC, Gust ID:** The relationship between a 27-nm virus-like particle and hepatitis A as demonstrated by immune electron microscopy. *Intervirology 4:110, 1974*

28. **Gravelle CR, Hornbeck CL, Maynard JE, Schable CA, Cook EH, Bradley DW:** Hepatitis A: Report of a common-source outbreak with recovery of a possible etiologic agent. II. Laboratory studies. *J Infect Dis 131:167, 1975*

29. **Provost PJ, Ittensohn OL, Villarejos VM, Hilleman MR:** A specific complement-fixation test for human hepatitis A employing CR326 virus antigen. Diagnosis and epidemiology. *Proc Soc Exp Biol Med 148:962, 1975*

30. **Krugman S, Friedman H, Lattimer C:** Viral hepatitis, type A: Identification by specific complement fixation and immune adherence tests. *N Eng J Med 292:1141, 1975*

31. **Miller WJ, Provost PJ, McAleer WJ, Ittensohn OL, Villarejos VM, Hilleman MR:** Specific immune adherence assay for human hepatitis A antibody. Application to diagnostic and epidemiologic investigations. *Proc Soc Exp Biol Med 149:254, 1975*

32. **Moritsugu Y, Dienstag JL, Valdesuso J, Wong DC, Wagner J, Routenberg JA, Purcell RH:** Purification of hepatitis A antigen from feces and detection of antigen and antibody by immune adherence hemagglutination. *Infect Immun 13:898, 1976*

33. **Hollinger FB, Bradley DW, Maynard JE, Dreesman GR, Melnick JL:** Detection of hepatitis A viral antigen by radioimmunoassay. *J Immunol 115:1464, 1975*

34. **Purcell RH, Wong DC, Moritsugu Y, Dienstag JL, Routenberg JA, Boggs JD:** A microtiter solid-phase radioimmunoassay for hepatitis A antigen and antibody. *J Immunol 116:349, 1976*

35. **Dienstag JL, Feinstone SM, Kapikian AZ, Purcell RH, Boggs JD, Conrad ME:** Fecal shedding of hepatitis A antigen. *Lancet 1:765, 1975*

36. **Dienstag JL, Routenberg JA, Purcell RH, Hooper RR, Harrison WO:** Foodhandler-associated outbreak of hepatitis type A: An immune electron microscopic study. *Ann Int Med 83:647, 1975*

37. **Evans AS:** Attempts to transmit the virus of human hepatitis to primates other than man. In "Symposium on the Laboratory Propagation and Detection of the Agent of Hepatitis," *Nat'l Acad Sci Nat Res Council Publ No. 322, Washington, D.C. 1954, p. 58-68*

38. **Hillis WD:** An outbreak of infectious hepatitis among chimpanzee handlers at a United States Air Force Base. *Am J Hyg 73:316, 1961*

39. **Barker LF, Chisari FV, McGrath PP, Dalgard DW, Kirschstein RL, Almeida JD, Edgington TS, Sharp DG, Peterson MR:** Transmission of type B viral hepatitis to chimpanzees. *J Infect Dis. 127:648, 1973*

40. **Maynard JE, Bradley DW, Gravelle CR, Ebert JW, Krushak DH:** Preliminary studies of hepatitis A in chimpanzees. *J Infect Dis 131:194, 1975*

41. **Dienstag JL, Feinstone SM, Purcell RH, Hoofnagle JH, Barker LF, London WT, Popper H, Peterson JM, Kapikian AZ:** Experimental infection of chimpanzees with hepatitis A virus. *J Infect Dis 132:532, 1975*

42. **Thornton A, Zuckerman AJ, Almeida, JD,** Chimpanzee model for hepatitis A virus (letter). *Lancet 2:226,1975*

43. **Schulman AN, Dienstag JL, Jackson DR, Hoofnagle JH, Gerety RJ, Purcell RH, Barker LF:** Hepatitis A antigen particles in chimpanzee liver, bile, and stool. *J infect Dis (in press) 1976*

44. **Deinhardt F, Peterson D, Cross G, Wolfe L, Holmes AW:** Hepatitis in marmosets. *Am J Med Sci 270:73, 1975*

45. **Purcell RH, Dienstag JL, Feinstone SM, Kapikian AZ:** Relationship of hepatitis A antigen to viral hepatitis. *Am J Med Sci 270:61, 1975*

46. **Feinstone SM, Kapikian AZ, Gerin JL, Purcell RH:** Buoyant density of the hepatitis A virus-like particle in cesium chloride. *J Virol 13:1412, 1974*

47. **Bradley DW, Hornbeck CL, Gravelle CR, Cook EH, Maynard JE:** CsCl banding of hepatitis A associated virus-like particles. *J Infect Dis 131:304, 1975*

48. **Hollinger FB, Bradley DW, Dreesman GR, Melnick JL:** Detection of viral hepatitis type A. *Am J Clin Path 65:854, 1976*

49. **Rowlands DJ, Shirley MW, Sangar DV, Brown F:** A high density component in several vertebrate enteroviruses. *J Gen Virol 29:223, 1975*

50. **Krugman S:** Hepatitis A virus; Effect of human immune serum globulin on infectivity. *J Infect Dis (in press) 1976*

51. **Feinstone SM, Kapikian AZ, Purcell RH, Alter HJ, Holland PV:** Transfusion-associated hepatitis not due to viral hepatitis type A or B. *N Eng J Med 292:767, 1975*

52. **Knodell RG, Conrad ME, Dienstag JL, Bell CJ:** Etiological spectrum of post-transfusion hepatitis. *Gastroenterology 69:1278, 1975*

53. **Alter HJ, Purcell RH, Holland PV, Feinstone SM, Morrow, AG, Moritsugu Y:** Clinical and serological analysis of transfusion-associated hepatitis. *Lancet 2:838, 1975*

54. **Dienstag JL, Gust ID, Lucas CR, Wong DC, Purcell RH,** Mussel-associated viral hepatitis, type A: Serologic confirmation. *Lancet 1:561 1976*

55. **Dienstag JL, Davenport FM, McCollum RW, Hennessy AV, Klatskin G, Purcell RH:** Nonhuman primate-associated viral hepatitis Type A: Serologic evidence of hepatitis A virus infection. *JAMA (in press), 1976*

56. **Mosley JW:** New patterns of transfusion-associated hepatitis. *Epatologica 12:527, 1966*

57. **Villarejos VM, Visona KA, Eduarte A, C.A., Provost PJ, Hilleman MR:** Evidence for viral hepatitis other than type A or type B among persons in Costa Rica. *N Eng J Med 293:1350, 1975*

58. **Galbraith RM, Portman B, Eddleston, ALWF, Williams R, Gower PE:** Chronic. liver disease developing after outbreak of HBs Ag-negative hepatitis in haemodialysis unit. *Lancet 2:886, 1975*

A CRITIQUE OF INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER (IASL) CRITERIA FOR THE DIAGNOSIS AND TREATMENT OF VIRAL HEPATITIS

Carroll M. Leevy

ABSTRACT. IASL standardization of diagnostic criteria and diagnostic methodology for viral hepatitis has reduced confusion resulting from multiple interpretations of data collected on patients with this disease and its clinical variants. It provides a basis for controlled studies urgently needed to evaluate the merits of new preventive, diagnostic, and therapeutic approaches for viral hepatitis.

A major diagnostic problem relates to the occurrence of hepatitis due to a virus other than "A" or "B." This new cause of hepatitis labeled virus "C" is particularly difficult to differentiate from drug-induced hepatitis. Serologic tests for alcoholic hyalin and histologic studies have facilitated its differentiation from alcoholic hepatitis.

Criteria for chronic and fulminant viral hepatitis do not reflect the prognostic implications of these diseases. Established criteria for chronic persistent and chronic active hepatitis should be flexible. Other diagnostic measures which objectively evaluate morphologic status and regenerative capacity are needed to properly assess proposed new methods of therapy for the fulminant disease.

Categorization of diagnostic methodology helps the practitioner decide which of the large number of tests should be used. Test category as illustrated by clearance studies changes depending upon its practicability, sensitivity, specificity and reliability.

Dr. Popper, Dr. Capps, Ladies and Gentlemen. I am delighted to be here to pay tribute to Richard Capps who as an expert clinician, educator and scientist has made many lasting contributions to both the science and art of hepatology and medicine. My report today is based on delineations of the International Association for the Study of the Liver (IASL), an organization of which Dr. Capps is a valued member. It was made possible, in part, by the innovative studies done in this university by Drs. Deinhardt, Capps and associates on viral A hepatitis.

Carroll M. Leevy, M.D., Chairman, Criteria Committee, International Association for the Study of the Liver (IASL); Director of the Divison of Hepatology and Nutrition, College of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103

Recent progress in our knowledge of viral hepatitis includes: detection of causative agents for "A" and "B" viral hepatitis by serologic and morphologic techniques; demonstration that immune globulins may prevent or modify these diseases; and elucidation of the effectiveness of various therapeutic regimens proposed for treatment of fulminant and chronic forms of viral hepatitis. Standardization of diagnostic criteria and methodology for viral hepatitis by the IASL permits controlled studies needed to test the efficacy of current and newly proposed diagnostic and therapeutic measures for viral hepatitis. Clinical, functional, morphologic and etiologic criteria for viral hepatitis and its variants have been established (Table I). Recommendations have also been made as to what

diagnostic methodology should be available, based on the simplicity, reliability, sensitivity, selectivity and specificity of a given process. Tests have been categorized into Level I which should be available in any medical facility; Level II which should be available in regional and university centers; and Level III which should be available in special liver centers (Table II). Discussions by the Criteria Committee have pinpointed problems which face clinicians caring for patients with viral hepatitis, and in many instances, investigations have been instituted to help resolve these problems. My discussion will focus on four of these problems: (a) establishing an etiologic diagnosis; (b) recognition and prognostication in fulminant viral hepatitis; (c) differentiation of chronic persistent from chronic active hepatitis; and (d) laboratory detection of subclinical liver injury and assessment of healing.

TABLE I.

FORMAT FOR IASL DIAGNOSTIC CRITERIA FOR DISEASES OF THE LIVER AND BILIARY TRACT.

(A) CLINICAL CRITERIA
(B) FUNCTIONAL CRITERIA
1. HEPATOCYTE
2. BILIARY
3. VASCULAR BED
4. IMMUNOCYTES AND MESENCHYMAL CELLS
5. TEST FOR METALS and NEOPLASIA
(C) MORPHOLOGIC CRITERIA
(D) ETIOLOGIC CRITERIA

TABLE II.

IASL CATEGORIZATION OF DIAGNOSTIC METHODOLOGY.

DETERMINANTS	
1. SENSITIVITY	4. RELIABILITY
2. SPECIFICITY	5. SIMPLICITY
6. SELECTIVITY	6. PRACTICALITY
CLASSIFICATION	
1. LEVEL I	— SHOULD BE AVAILABLE IN ANY MEDICAL FACILITY
2. LEVEL II	— AVAILABLE IN UNIVERSITY OR REGIONAL HOSPITAL CENTERS
3. LEVEL III	— AVAILABLE IN SPECIALIZED CENTERS FOR DIAGNOSIS AND TREATMENT OF HEPATIC AND BILIARY TRACT DISORDERS

ETIOLOGY OF HEPATITIS

Controlled investigations by several groups indicate that an increasing number of patients have hepatitis indistinguishable from that due to virus "A" or "B" in which serologic tests for these agents are negative. Such hepatitis which has been tentatively designated as "C", usually has a long incubation period. A cooperative Veterans Administration Post-transfusion Hepatitis Study in which our group in New Jersey has participated supports the concept of another etiologic agent for viral hepatitis, although much further information is needed on its incidence, nature and sequelae. Despite elimination of HB_sAg positive donor blood and the use of pooled gamma globulin to protect against viral "A" hepatitis, the in-

cidence of post transfusion hepatitis initially reduced by exclusion of HB positive donor blood has not changed significantly in urban areas using commercial blood, despite routine blood screening. We have interpreted this as evidence that a non "A" and "B" form of viral hepatitis is being transmitted by transfused blood. This thesis is confirmed by recent studies which show such patients have morphology typical of viral hepatitis in the absence of other causes of hepatitis or positive serologic tests for HAAg, HAAb, HB_sAg, HB_cAg, HB_sAb or HB_cAb.

Existence of viral hepatitis which cannot be identified by a marker returns us to the pre-hepatitis "B" antigen days in some patients, since it is often difficult to differentiate this from other types of hepatitis. Drug-induced hepatitis is illustrative. Of 25 patients seen at the New Jersey Medical School with histological and clinical features compatible with viral hepatitis and/or drug-induced hepatitis, 15 were HB_sAg positive and 10 HB_sAg negative. Circumstantial evidence permitted a diagnosis of concurrent drug-induced hepatitis in 5 of the antigen positive patients. In the 10 HB_sAg negative patients, the true cause of their hepatitis remains unknown since no marker for viral or drug-induced hepatitis was or is yet available (Fig. 1).

An equally thorny problem is presented in differentiating viral from alcoholic hepatitis. The alcoholic frequently develops viral and/or drug hepatitis, and it is important to decide, for therapeutic purposes, what is responsible for the liver injury. Recognition of alcoholic hepatitis has been facilitated by the detection in serum of an antigen to alcoholic hyalin in early phases of the disease followed by the appearance of antibodies to this glycoprotein which has been isolated and partially purified. Recurrent hepatitis is associated with reappearance of the antigen. Of 50 alcoholics with a history of drug abuse, 25 had serologic evidence of alcoholic hyalin for antigen or antibody; 15 were HB antigen positive, and in the remainder, neither serologic nor histologic studies permitted a definite etiologic diagnosis (Fig. 2). Again, development of appropriate methods for detection of virus "C" would be helpful since two etiologic factors may be present.

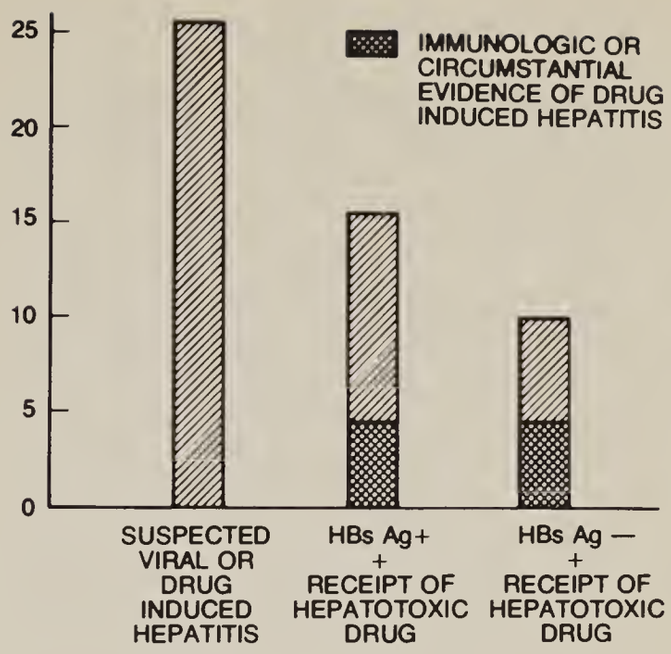


Fig. 1—Differentiation of viral from drug-induced hepatitis.

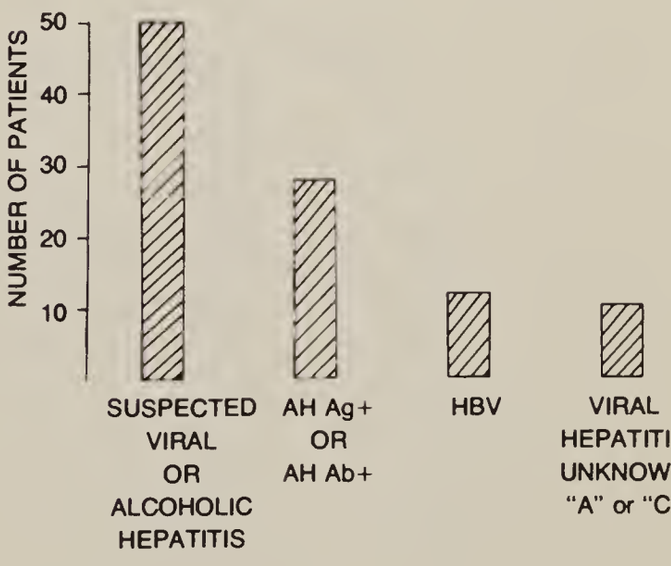


Fig. 2—Differentiation of viral from alcoholic hepatitis.

FULMINANT VIRAL HEPATITIS

A correlation of IASL criteria provides new therapeutic orientation for fulminant viral hepatitis. According to adopted criteria, this designation is appropriate when there is sufficient liver necrosis to cause impairment of synthesis of liver derived protein (e.g., prothrombin), and failure to metabolize or detoxify compounds capable of causing encephalopathy. Controlled studies show that no currently available therapeutic modality has had a significant influence on survival.

Approximately 20 to 25 per cent of patients who have fulminant viral hepatitis according to these criteria survive. In our experience, 80 per cent of those who survive eventually exhibit normal liver histology, and 20 per cent have chronic active hepatitis (Fig. 3). In predicting prognosis, liver histology, bleeding tendency, severity of mental changes, electrolyte balance and renal function have been helpful but not specific. Opolon and associates in France have attempted to determine prognosis by sterological counts of hepatocytes obtained by percutaneous liver biopsy during the initial phase of hospitalization. It was found that there is a good correlation between hepatocyte volume and recovery of consciousness as well as survival. All patients with hepatocyte volume of less than 35 per cent died, whereas, 20 per cent of those with hepatocyte volume of more than 35 per cent survived. This has been interpreted as evidence that prognosis is directly related to the severity of the initial injury. These and other observations indicate the need to supplement IASL criteria with information on hepatocyte volume, regenerative capacity and other factors to properly classify patients with fulminant hepatitis. Only then will it be possible to conduct urgently needed controlled studies on the value of artificial assists and other therapies proposed to improve survival in this condition.

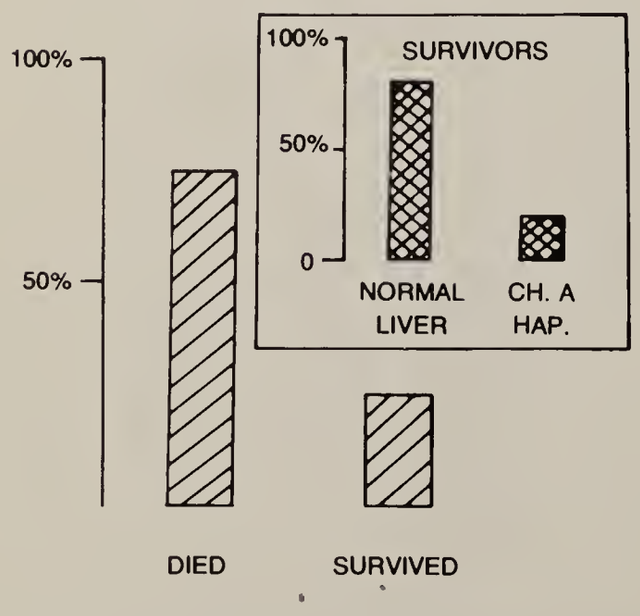


Fig. 3—Fulminant viral hepatitis.

CHRONIC HEPATITIS

Differences in nomenclature and classification of chronic viral hepatitis in various countries and within the United States have prevented a comparison of treatment results in this condition. IASL criteria for chronic persistent and chronic active hepatitis have eliminated much of existing overlap. However, nomenclature still leaves much to be desired. Diagnostic criteria are primarily based on histology. Hepatitis must be present for six or more months to receive a designation of chronic. Patients with chronic active hepatitis which warrant special treatment exhibit piecemeal necrosis eroding the limiting plate, bridging necrosis or multitubular necrosis. Patients with persistent hepatitis have inflammatory round cell infiltration of portal areas with absent or minimal piecemeal necrosis and occasional focal necrosis. Serial biopsies and clinical features com-

patible with either disease may show the other lesions subsequently, owing to sampling or change in status.

Serial studies of a group of patients with the designation of chronic persistent hepatitis over a five-year period in New Jersey revealed one-half had stabilization of histologic and biochemical abnormalities; 30 per cent had a disappearance of all abnormalities. Unexpectedly, 3 or 5 per cent developed fulminant hepatitis following the stress of surgery or pregnancy (Fig. 4).

TABLE III.

COURSE OF HBsAG POSITIVE PATIENTS WITHOUT HEPATITIS.

PATTERN A (5)	+	+	+	+
PATTERN B (3)	+	+	+	—
PATTERN C (4)	+	+	—	—
	1	2	3	4 YEARS

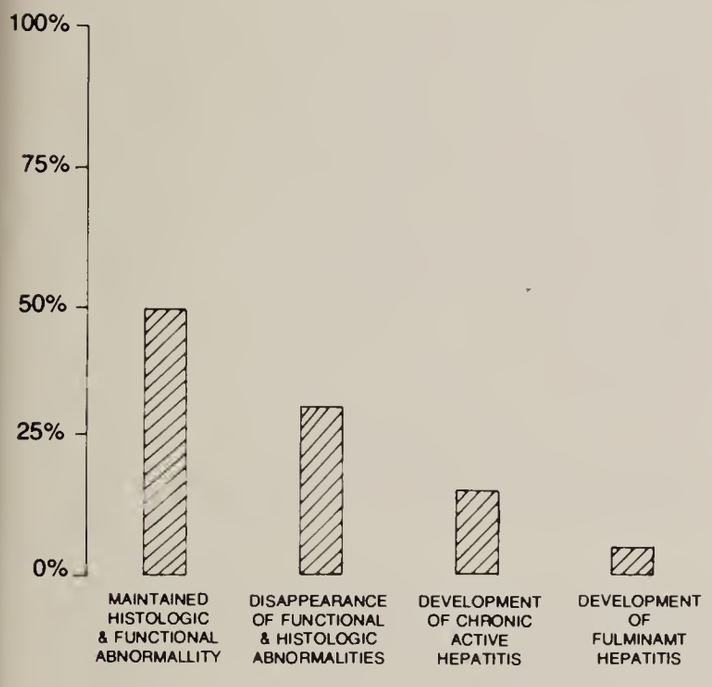


Fig. 4—Natural history of chronic persistent viral hepatitis.

Closely related is the problem of the person with persistent HBsAg without functional or morphologic evidence of liver injury. In these instances, an occasional person develops hepatitis. After two to four years many such patients become HBsAg negative (Table III), so that permanent change of occupation may not be necessary.

In considering chronic hepatitis, the IASL Criteria Committee focused much attention on immunologic studies in the belief that immunologic reactivity to the hepatitis virus or damaged liver is responsible for chronicity. This hypothesis is supported by studies of cell mediated immunity which demonstrate blastogenic, migration inhibition, cytotoxic and transfer factors. Addition of HBsAg to lymphocytes from patients with either acute or chronic viral "B" hepatitis regularly produces an increase in the stimulation index and a decrease in the migration inhibition index (Fig. 5). This is interpreted as evidence that immunologic reactivity to the virus or its product is principally responsible for liver cell destruction in acute hepatitis.

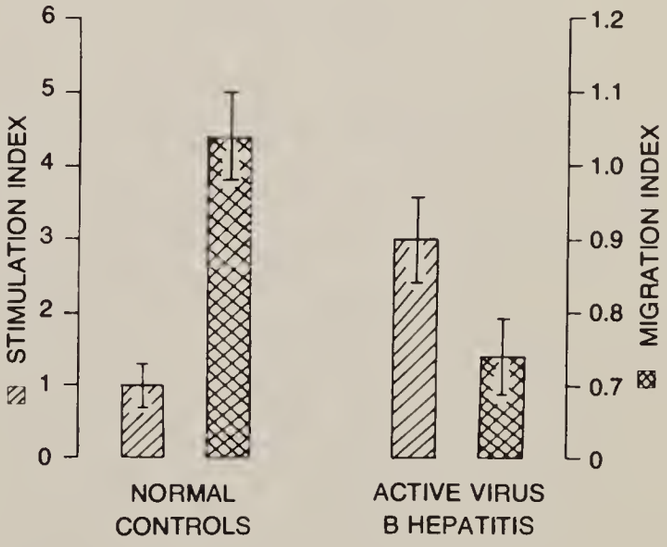


Fig. 5—Influence of HBsAg on reactivity of lymphocytes in acute viral hepatitis.

It has been postulated that overall immunocompetence may also be of key importance in determining the nature and course of viral hepatitis and the desirability of assessing the same in complicated cases. Both acute and chronic viral hepatitis are characterized by a decrease in circulating T cells which return to normal with recovery. Acute viral hepatitis is characterized by a significant decrease in lymphocyte reactivity to phytohemagglutinin (PHA) and other non-specific antigens. This diminution in reactivity which returns to normal with recovery may be due to lymphocyte injury, a circulating antagonist or nutrient deficit. Chronic active hepatitis is also associated with a decrease in PHA responsive lymphocytes. Some believe reduced reactivity is responsible for chronicity, but in our experience, PHA response returns to normal with recovery, so that special host alteration of this type would not appear to be responsible for chronicity. More probably, the hyperactivity of lymphocytes sensitized to hepatitis viral antigens and damaged liver (? liver specific protein) cause continuing destruction of hepatocytes characteristic of this disease.

DIAGNOSTIC ASSESSMENT OF HEALING

Categorization of clinical tests for evaluating patients with hepatic disorders into Levels I, II and III has constituted a unique and important decision by the IASL Criteria Committee. This provides the practitioner with information on what tests should be ordered and what to expect of these tests in diagnostic evaluation of patients with viral hepatitis. It also gives information on sophisticated diagnostic tests which are available in regional and in special centers with a special interest in the liver and its diseases. A new dimension is provided which, if extended to other organ diseases, will revolutionize the practice of medicine.

Included in Level I tests (Table IV) are the simple biochemical and serologic tests commonly used for diagnosis of viral hepatitis. Also included is liver biopsy. Previous resistance to liver biopsy has been dispelled by the extremely low morbidity from the procedure. Biopsy is desirable for proper workup of patients with sporadic viral hepatitis to determine its nature, activity and chronicity. It also is essential for the differentiation of other forms of hepatitis, particularly in the absence of a marker for viral "C" hepatitis at this time. Special techniques including special stains, electron microscopy, autoradiography and chemical analysis of biopsies are categorized as Levels II and III.

TABLE IV.

IASL LEVEL I TESTS FOR VIRAL HEPATITIS

1. AMINO — TRANSFERASES
2. SERUM PROTEINS
3. PROTHROMBIN TIME
4. DYE RETENTION
5. SERUM BILIRUBIN and FRACTIONATION
6. SERUM ALKALINE PHOSPHATASE
7. LIVER BIOPSY
8. VIRUS B_S ANTIGEN and ANTIBODY DETECTION
   (a) IMMUNODIFFUSION
   (b) COUNTER IMMUNOELECTROPHORESIS
   (c) INERT PARTICLE AGGLUTINATION

Level II tests (Table V) include more sophisticated biochemical and serological tests. Simple clearance tests have also been included in this category, since, at least theoretically, they offer the best approach to quantification of liver reserve.

TABLE V.

IASL LEVEL II TESTS FOR VIRAL HEPATITIS.

1. ICG CLEARANCE
2. SERUM 5′ NUCLEOTIDASE
3. SERUM BILE ACIDS
4. SERUM IMMUNOGLOBULINS
5. ALPHA FETO PROTEIN
6. HB_sAg and HB_sAb DETECTION
   (a) COMPLEMENT FIXATION
   (b) RADIOIMMUNOASSAY
   (c) ELECTRON MICROSCOPY

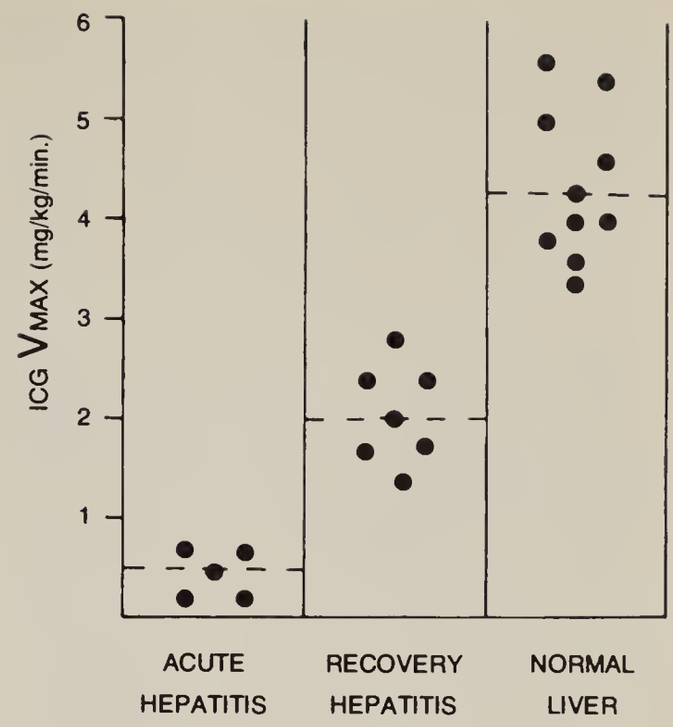


Fig. 6—ICG Vmax in viral hepatitis.

Maximum hepatic removal of a test substance which may be calculated according to Michaelis-Menten kinetics is dependent on blood flow, membrane transport, enzymatic utilization of substrate and excretory function. A variety of exogenous substances including indocyanine green, bile acids, drugs and bilirubin have been used as test agents. Their safety and practical usefulness is related to toxicity and ease of measuring disappearance rate.

Using the clearance principle, a study of removal of indocyanine green (ICG) and measurement of post prandial serum bile acids are currently the most popular tests for screening patients with viral hepatitis. ICG exhibits negligible toxicity; it is not disposed of by extrahepatic means and its removal may be reliably evaluated by dichromatic ear densitometry which permits non-invasive mass screening of patients. Patients with acute hepatitis and recovery phase hepatitis regularly have significant reduction in ICG removal capacity and calculated maximal rate of clearance (Vmax) (Fig. 6).

Measurement of bile acid conjugates postprandially could provide equal information. Glycine conjugated bile acids diffuse into portal vein blood after the gallbladder is evacuated following a meal, and are then extracted by the liver so that serum bile acids return to normal two hours after eating. Acute hepatitis is characterized by significant increase in postprandial bile acids, and a similar phenomenon is often, but not always, present in recovery phase hepatitis when bile acids are measured chemically (Fig. 7). In a compari-

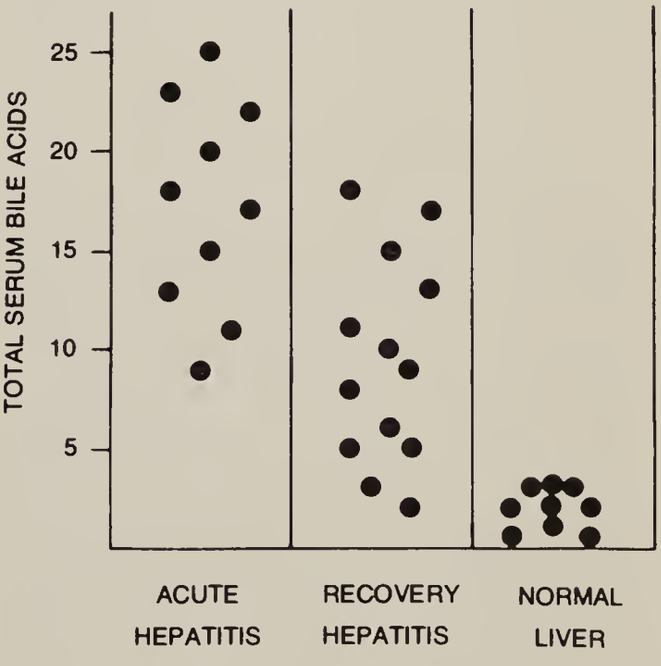


Fig. 7—Two hour post prandial bile acids in viral hepatitis.

son of the sensitivity of ICG and postprandial bile acids, our data to date indicate that ICG is a more sensitive index than postprandial serum bile acids when the latter are measured by gas liquid chromatography and less accurate chemical methods. ICG has been regularly abnormal when postprandial bile acids are elevated and this abnormality persists when the postprandial bile acids become normal (Fig. 8).

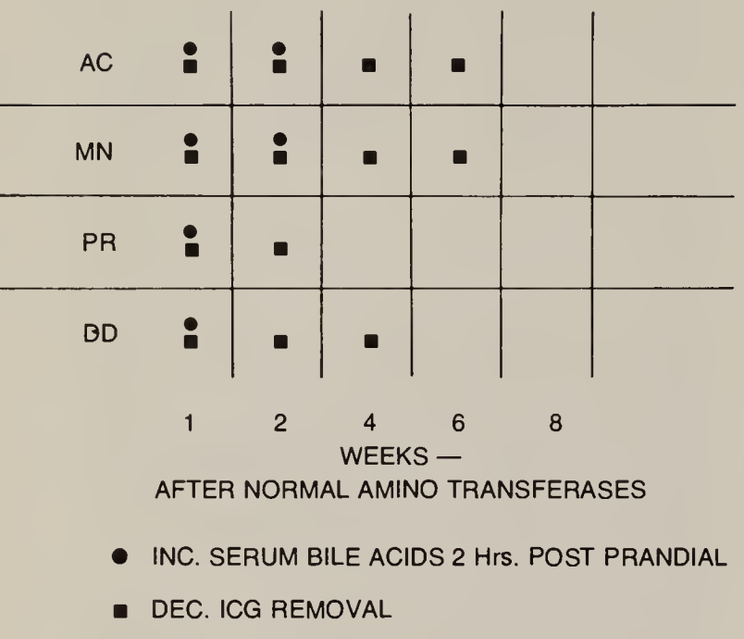


Fig. 8—Comparison of ICG removal rate (5 mg per kilo) and 2 hour post prandial bile acids in recovery phase of viral hepatitis with normal aminotransferases.

Categorized as Level III tests are clearance of galactose and bile acids using radioactive preparations, although in both theory and practice it is not probable that these test agents will provide a more sensitive index to subclinical viral hepatitis than ICG. Sophisticated tests for identification of virus "A" and "B" hepatitis are included in this Level III category (Table VI), with the expectation, however, that they may eventually be reclassified as Category I or II with more experience or simplification of techniques. The IASL has

TABLE VI.

IASL LEVEL III TESTS FOR VIRAL HEPATITIS

1. GALACTOSE ELIMINATION CAPACITY
2. BILE ACID CLEARANCE
3. LYMPHOCYTE RESPONSE TO HB_s Ag
4. HB_c and HB_cAb DETECTION
5. HEPATITIS A VIRUS
   (a) COMPLEMENT FIXATION
   (b) IMMUNE ADHERENCE TEST
   (c) FECAL VIRAL PARTICLES

agreed continuously to review its standardization and to work closely with the World Health Organization in this endeavor. The Criteria Committee will attempt to modify concepts as new discoveries are made to improve diagnostic measures which should eventually aid in prevention and treatment, and thereby reduce further morbidity and mortality from viral hepatitis.

REFERENCE

Diseases of the Liver and Biliary Tract, Standardization of Nomenclature, Diagnostic Criteria and Diagnostic Methodology, Fogarty International Center Proceedings No. 22, U.S. Government Printing Office, 1976

THE STUDY OF HEPATIC STRUCTURE IN VIRAL HEPATITIS

Fenton Schaffner

Study of hepatic structure continues to play an important role in the unravelling of the pathogenetic mechanisms of various forms of hepatic injury, particularly viral hepatitis, and in resolving practical problems in diagnosis. This presentation deals with some of the newer morphological information useful in the practical management of hepatitis and in the research laboratory.

STRUCTURAL CHANGES IN ACUTE HEPATITIS

The single most important step in the modern study of hepatitis was the development of needle biopsy technique. This permitted sampling of the still living liver, and description of the typical spotty necrotic lesions of viral hepatitis.[1] Single hepatocytes, or small groups of them, undergo liquefaction necrosis which elicits a focal inflammatory response and portal inflammation (Fig. 1).[2] The necrosis is scattered throughout the lobule with some centrolobular predominance.[3] The walls of the central veins are edematous and contain scattered macrophages and other inflammatory cells. Occasional hepatocytes undergo coagulation necrosis. Electron microscopic study was required to recognize that the acidophilic "Councilman-like" bodies are dead hepatocytes (Fig. 2).[4]

Fig. 1—Liver biopsy specimen from patient with acute viral hepatitis. Small clusters of inflammatory cells (spotty necrosis) are scattered about the parenchyma, and the central vein in the center shows infiltration of its wall in places with the same cells *(H & E 100)*.

The development of fixation, embedding and cutting techniques for use with electron optics extended the morphologists' range of vision. They could now recognize what was wrong with the surviving hepatocytes, important because the extent of their malfunction usually determines the severity of illness. While hepatocytic cell mass was found to be decreased by shedding of pieces of

From the Division of Liver Diseases, Department of Medicine, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029

Fenton Schaffner, M.D., Gerge Baehr Professor of Medicine and Chief, Division of Liver Diseases, Mount Sinai School of Medicine

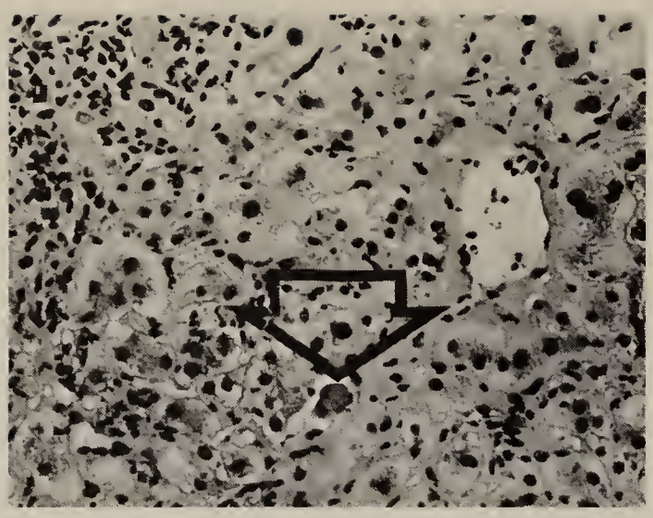


Fig. 2—Acidophilic (Councilman-like) body (arrow) in acute viral hepatitis. The inflammatory exudate in the upper left corner is spilling out of a nearby portal tract *(H & E X 250)*.

cells, cellular autophagy and necrosis, hepatic dysfunction was chiefly associated with disruption of the endoplasmic reticulum.[5] Alteration of this organelle is responsible for loss of cytoplasmic basophilia through dispersion of ribosomes from rough profiles (Fig. 5), and for part of the hydropic swelling because of vesiculation and distension of the profiles by fluid.

Death of all cells in the liver was long known to result in fatal massive necrosis,[6] and the common clinical picture of acute, self-limited hepatitis is characterized by spotty necrosis. Some patients are more sick than average or are sick much longer. Study of liver biopsy specimens from this group indicate that more than scattered cells have undergone necrosis and that cells have died in a line extending from the portal tract to the central vein.[7] This bridging necrosis also often presages chronic hepatitis and cirrhosis, although healed bridges can be recognized in the absence of residual disease. Rarely is necrosis so extensive that all cells in a lobule or in a contiguous group of lobules perish.[8] The framework collapses; and a broad irregular scar results. When such areas of collapse are few, they have no functional significance but when widespread, they deform the liver to produce true postnecrotic cirrhosis. Thus study of the liver aids in prognosis in acute hepatitis.

STRUCTURAL CHANGES IN CHRONIC HEPATITIS

Concern with prognosis is even greater when hepatitis is protracted or when hepatic dysfunction begins insidiously. It is probably here that the liver biopsy procedure has its widest use today. Chronic hepatitis implies extended duration, and the most widely accepted length of illness applicable to the term chronic is six months. In those patients with insidious onset, the precise delineation of the time factor is not possible, and morphologic guidelines are more important.[9,10] Spotty necrosis may continue in the lobular parenchyma along with portal inflammation for many months (Fig. 3). Often this protracted acute *lobular hepatitis* heals, but since it may progress to more serious chronic disease, accurate prognostication is not possible. As hepatitis heals, but before results of tests of hepatic function return to normal, nonspecific structural abnormalities persist. These include small histocytic nodules *(Spätknötchen)* (Fig. 4), focal necrosis, and portal inflammation. *Nonspecific hepatitis* implies that healing is nearly complete. By contrast, the lobular changes may subside and inflammation may persist only in or in and around the portal tracts. *Chronic portal hepatitis* is recognized as the clinical entity of chronic persistent hepatitis.[11] The inflammation which had extended from the portal tracts into the parenchyma in the acute stage retracts to within the portal tracts, allowing the limiting plates of hepatocytes to restore themselves. This lesion heals

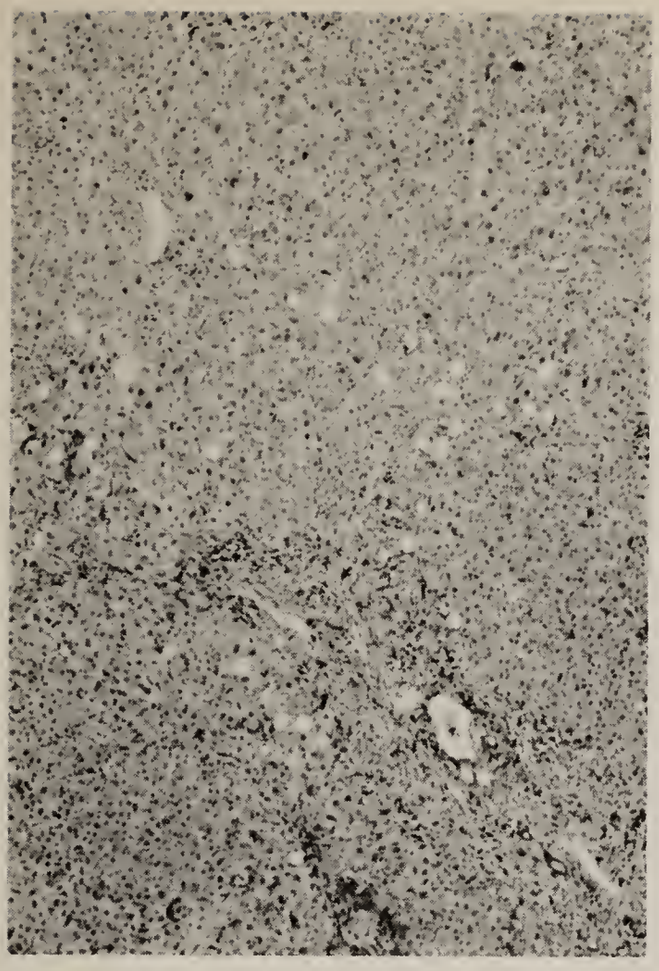


Fig. 3—Protracted acute or lobular hepatitis more than 8 months after the onset of disease. Spotty necrosis is still scattered throughout *(H & E X 40.)*

without therapy in most cases, although progression to more serious disease occurs in a small percentage. When the lobular component of acute hepatitis subsides, but the portal and periportal inflammation does not, the resulting *chronic periportal hepatitis* is called aggressive. The loss of hepatocytes in the limiting plate, with resulting inflammation and subsequent fibrosis, is piecemeal necrosis, the *sine qua non* of chronic active hepatitis (Fig. 6). Bridges and collapse seen in acute hepatitis may persist or may develop anew from the chronic periportal hepatitis (Fig. 7). This form may spontaneously heal, but because it does so in a minority of cases, and is far more likely to progress to cirrhosis, active therapy is indicated. Thus study of structure offers guidelines for therapy as well as for prognosis.

RECOGNITION OF ETIOLOGY OF HEPATITIS

The clinical syndrome of hepatitis has many causes. Sometimes these can be recognized by the appearance of the biopsy specimen.[12] This is best exemplified by alcoholic hepatitis with its central hyalin necrosis and sclerosis (Fig. 8).[13] Acute hepatitis in addicts may be viral, alcoholic, or both, and clarification sometimes can only be obtained by liver biopsy. For instance, cells containing alcoholic hyalin may undergo coagulation necrosis and become acidophilic bodies, attesting to the dual etiology of the hepatitis. More importantly, much attention has been devoted to the description of drug-induced hepatitis. In some instances clues can be found in liver biopsy specimens to suggest a drug etiology rather than a viral one.[14] These clues include small granulomas such as occur with halothane hepatitis, or necrotizing cholangitis seen with chlorpropamide use, and, recently in one instance, with acetohexamide. Electron microscopic studies in halothane hepatitis suggest that in this condition mitochondrial injury may be in the foreground when compared to viral

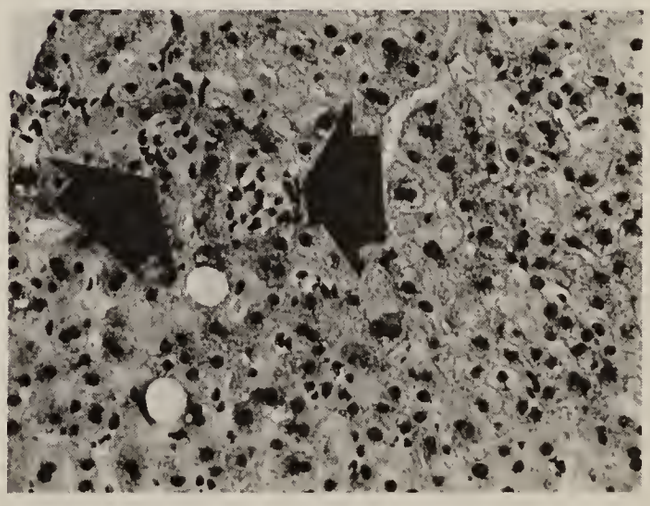


Fig. 4—Small nodules or *Spatknotchen* (arrows) in parenchyma in patient convalcesing slowly from acute viral hepatitis *(H & E X 250)*.

hepatitis.[15] The acute hepatitis caused by drugs like methyldopa or isoniazides cannot be readily distinguished by morphologic means from viral hepatitis, nor can the chronic hepatitis these drugs produce be separated from chronic hepatitis after acute viral hepatitis."[14]

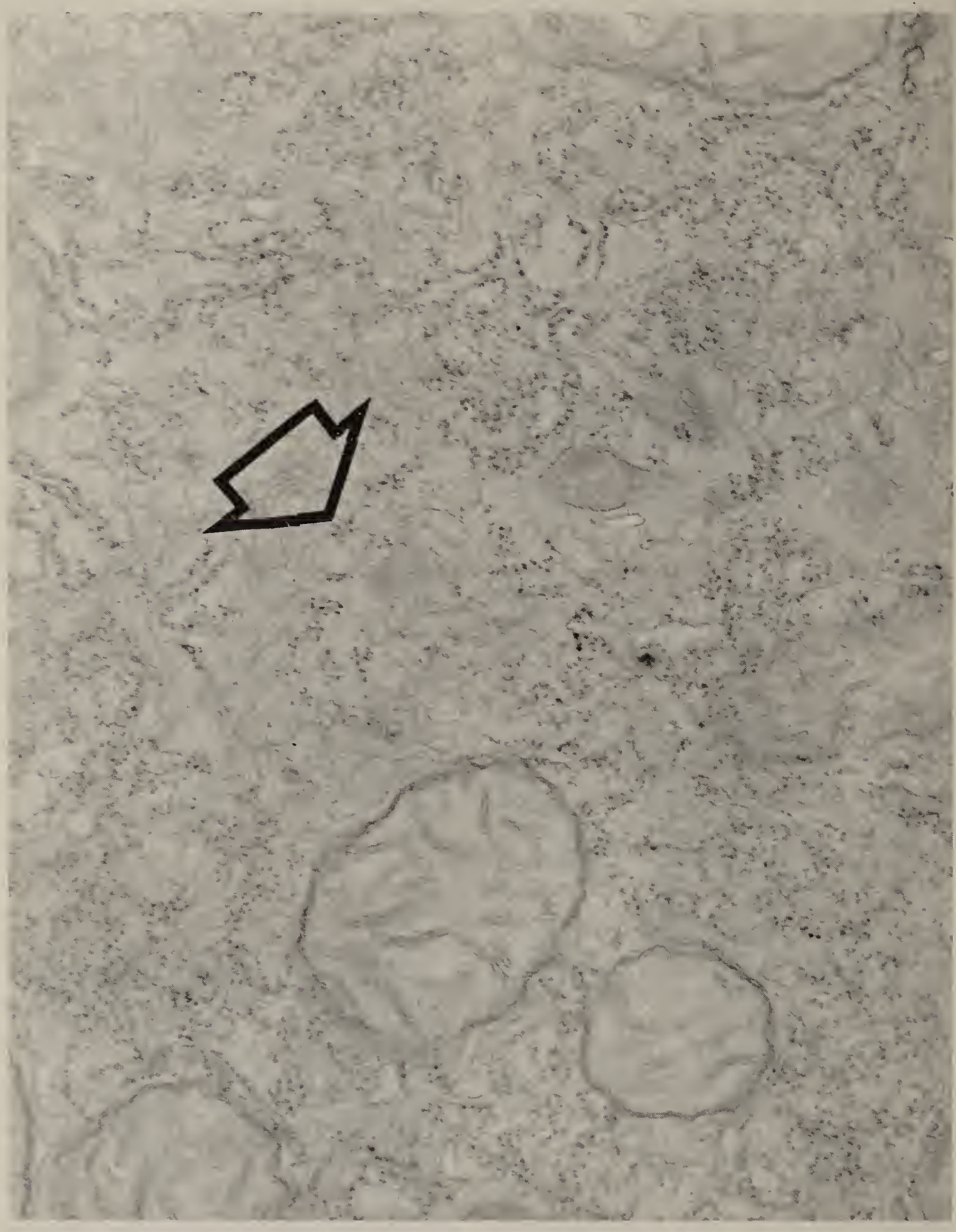


Fig. 5—Electron micrograph of liver biopsy specimen from patient with acute hepatitis. The numerous small dots are ribosomes and some are free in small clusters in the cytoplasm. The mitochondria in the lower portion of the picture are normal. On either side of the arrow remnants of rough profiles of endoplasmic reticulum are seen. In both lower corners the ribosomes are in small spirals (polysomes) unattached to membranes (osmium fixed, lead and uranyl stained X 40000).

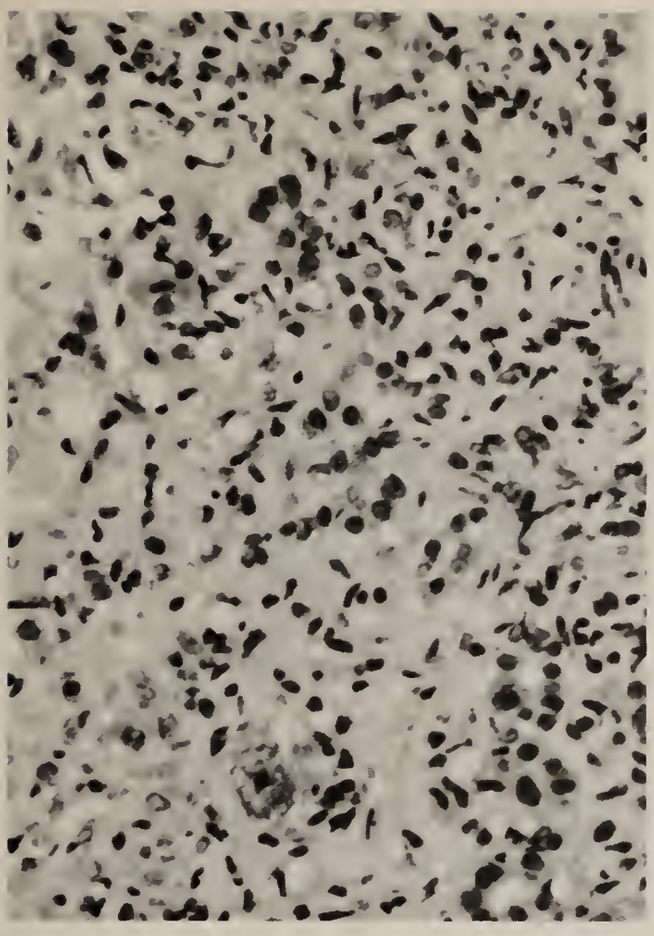


Fig. 6—Indistinct borderzone between a portal tract (right) and parenchyma (left) because of necrosis and inflammation characteristics of periportal "piecemeal" necrosis (H & E X 400).

HEPATATIS B VIRUS IN TISSUE

Hepatocytes containing hepatitis B surface antigen (HB_sAg) can be recognized in routine liver biopsy specimens in some circumstances. The cells are larger than normal because the bulky cytoplasm has a smudged appearance similar to ground or frosted glass (Fig. 9).[16] These cells are usually in small groups, with no zonal predilection. Ground glass hepatocytes were first noted in asymptomatic carriers of hepatitis B whose serum contained HB_sAG. They also have been seen in HB_sAg positive chronic hepatitis when diffuse liver cell injury is not severe. Originally such cells were noted in animals in which the hepatocellular microsomal biotransformation system was induced by drugs like phenobarbital. The appearance is due to hypertrophy of smooth endoplasmic reticulum in either case. The ground glass hepatocytes contain in the profiles of their endoplasmic reticulum the round 22 nm particles and the elongated rods that characterize HB_sAg in serum.[17,18] The particles are made in the endoplasmic reticulum, since immunofluorescent studies[19] and immunoelectron microscopy[17] have shown specific antigenic protein to be present in and around the membranes as well as in the particles. Paraffin-embedded sections can be stained with aldehyde fuchsin, orscein or thionine, and the ground glass cells are then uniquely visualized under light microscopy.[10,21,22] HB_sAg containing cells can be seen in tissue fixed over half a century ago.

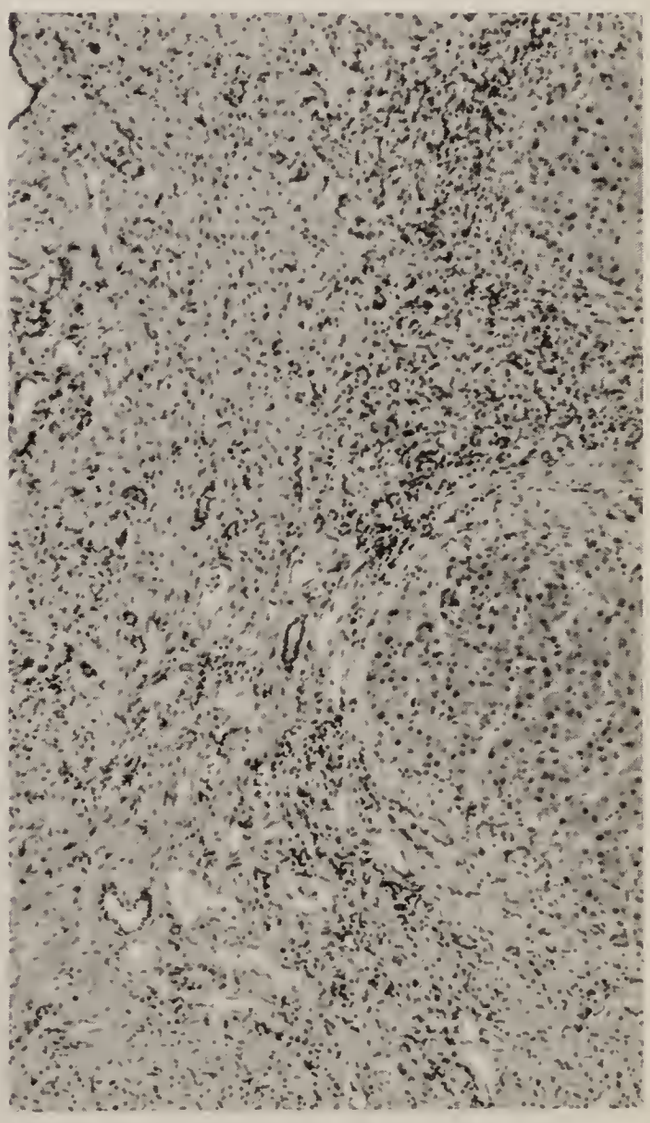


Fig. 7—Collapse of parenchyma in chronic active hepatitis. Two islands of liver cells on the right is all that remains of parenchyma while the reminder is collapsed stroma (H & E X 100).

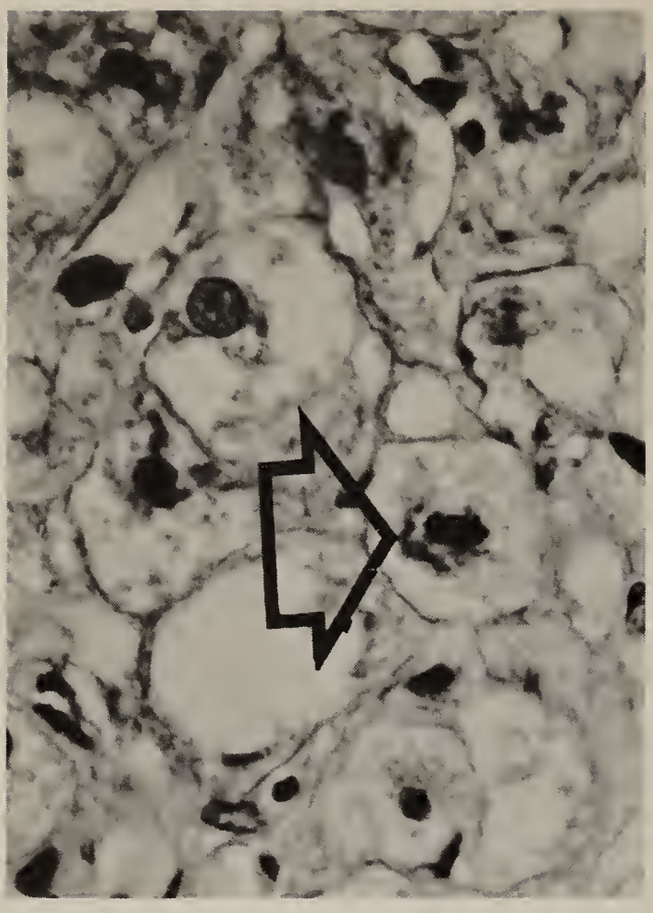


Fig. 8—Alcoholic hyalin of Mallory (arrow) in patient with alcoholic hepatitis (H & E X 400).

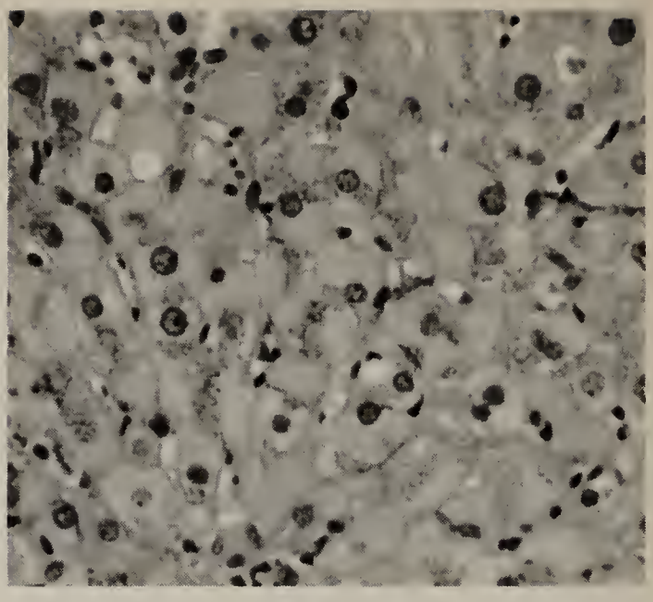


Fig. 9—Ground glass hepatocytes in carrier of hepatitis B antigen (H & E X 250).

CHOLESTASIS IN HEPATITIS

Cholestasis is a phenomenon deserving special consideration in the study of hepatic structure in hepatitis. Impairment of bile formation and flow occurs in almost all cases of hepatitis, and it usually is severe enough to produce jaundice. Cholestasis may not be recognized in routine liver biopsy specimens early in the course of hepatitis because the effects of diffuse hepatocytic injury overshadow it both morphologically and functionally.[23] As the hepatocytes recover, their ability to secrete bile lags behind, and centrolobular cholestasis may manifest itself clinically and histologically later in the disease. If the canaliculi are examined early by electron microscopy, cholestasis is apparent at that time. Cholestasis may be the predominant feature throughout the course of the disease. This is more likely to occur in older patients, and the more cholestasis there is, the longer the patient remains jaundiced. Biopsies taken late in the course of cholestatic hepatitis may reveal only centrolobular bile plugs, bile stained Kupffer cells and vacuolated bile stained hepatocytes (feathery degeneration) after all spotty necrosis and portal and periportal inflammation have subsided. Since the differential diagnosis of cholestasis is best made by newer techniques such as endoscopic retrograde or skinny needle cholangiography or by computerized axial tomography, great concern over the morphologic differential diagnosis of cholestasis is no longer necessary. As a matter of fact, liver biopsy should not be considered the procedure of choice for the differential diagnosis of cholestasis under any circumstances. Morphology has been replaced by radiologic methods to determine the cause of cholestasis.

PATHOGENESIS OF HEPATITIS

Hepatitis B: The study of hepatic structure is a useful tool for investigating the pathogenesis and evolution of viral hepatitis. The development of current techniques

has depended on the discovery of hepatitis B virus, its components, and the antibodies to different components. Mention has been made of the localization electron microscopically and immunologically of HB_sAg in the hepatocellular cytoplasm. The core particle of the virus can be localized in hepatocytic nuclei, particularly in immunosuppressed persons.[24] Immune globulins are found in some of these nuclei also, possibly core antibody (HB_cAg). The tissue localization, mainly by immunofluorescence, has led to hypotheses concerning the mechanism of hepatocellular injury in acute and chronic hepatitis.[25,26] These theories suggest that the plasma membranes of hepatocytes are altered by the virus, by viral components, or by abnormal proteins manufactured by the infected cell. The immune system of the host then eliminates the virally altered cells or injures them. Both humoral and cell mediated systems have been implicated in this complex interaction between the virus, hepatocytes and immunocytes. Modern morphologic methods are uncovering details of the interaction. The development of a laboratory model in the chimpanzee infected with hepatitis B will aid in these studies.[27] Core particles have been seen in the nuclei of the hepatocytes of infected chipanzees.

Hepatitis A: Similarly, the development of an animal model of hepatitis A in chimpanzees[28] and in tamarin monkeys[29] may advance our knowledge of the pathogenesis of hepatitis A, now that the virus has been identified and techniques

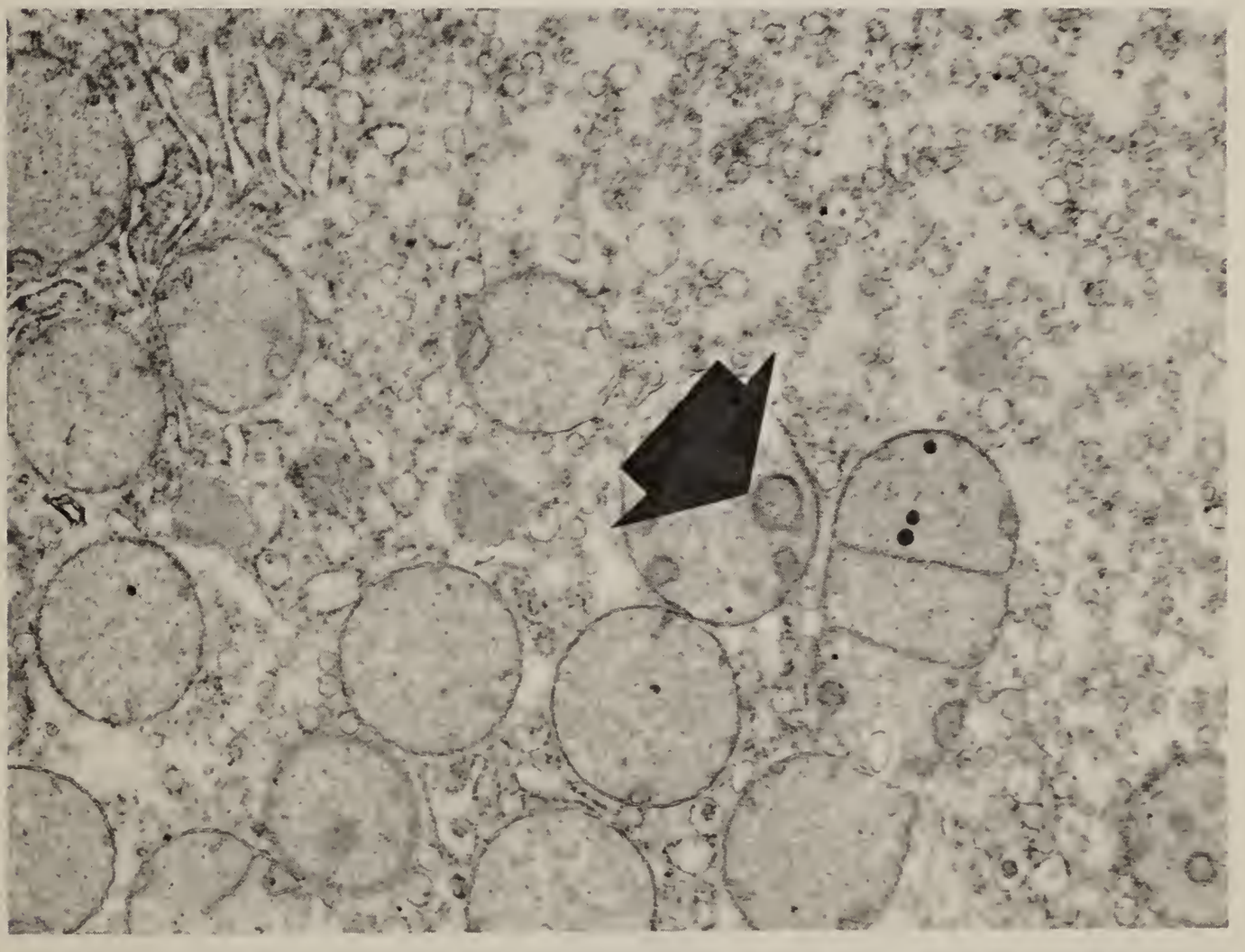


Fig 10—Mitochondria in liver of chimpanzee infected with hepatitis A showing enlarged mitochondria with transverse or curled or circular (arrow) cristae (glutaraldehyde-osmium fixed, lead-uranyl stained X 2500).

developed to measure antibody response. When chimpanzees infected with hepatitis A are examined, changes are found in mitochondria (Fig. 10) and in endoplasmic reticulum (Fig. 11) not seen in animals with hepatitis B.[30] Furthermore, large clumps of granules (Fig. 12), probably heterochromatin for synthesis of virus A RNA are found at the peak of disease. By contrast with virus B infection, changes seen in hepatitis A last only a week or two and then normalcy is restored. Immunofluorescence and immunoelectron microscopic studies are now needed to delineate the meaning of some of the findings.

FUTURE OF MORPHOLOGY

The outlook for the future of the study of liver tissues in viral hepatitis is a bright one which hopefully will soon include the development of antiviral chemotherapy against hepatitis A and hepatitis B as well as solve the mystery of the identity of non A-non B hepatitis. The entire question of the pathogenesis of virally induced cellular injury will be answered with the help of morphology. This should also lead to understanding of the mechanism of drug induced cellular injury, because the similarities between this and virally induced injury suggest that some common pathways exist possibly via the plasma membrane and the immune response of the host.

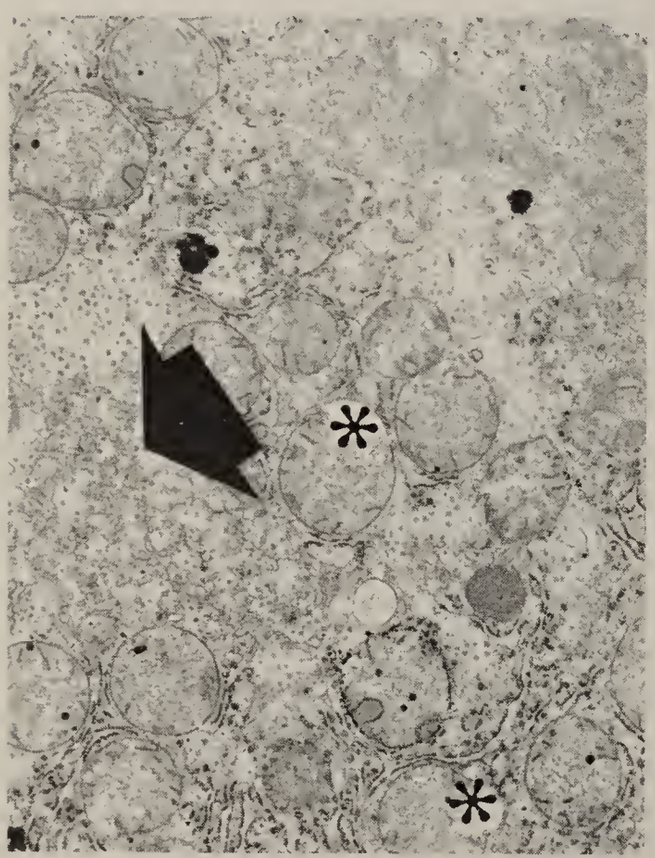


Fig. 11—Portion of hepatocyte from chimpanzee with hepatitis A showing clumped endoplasmic reticulum (arrow) and abnormal mitochondria as in Figure 10. The asterisks mark fixation artifacts (X 10000).

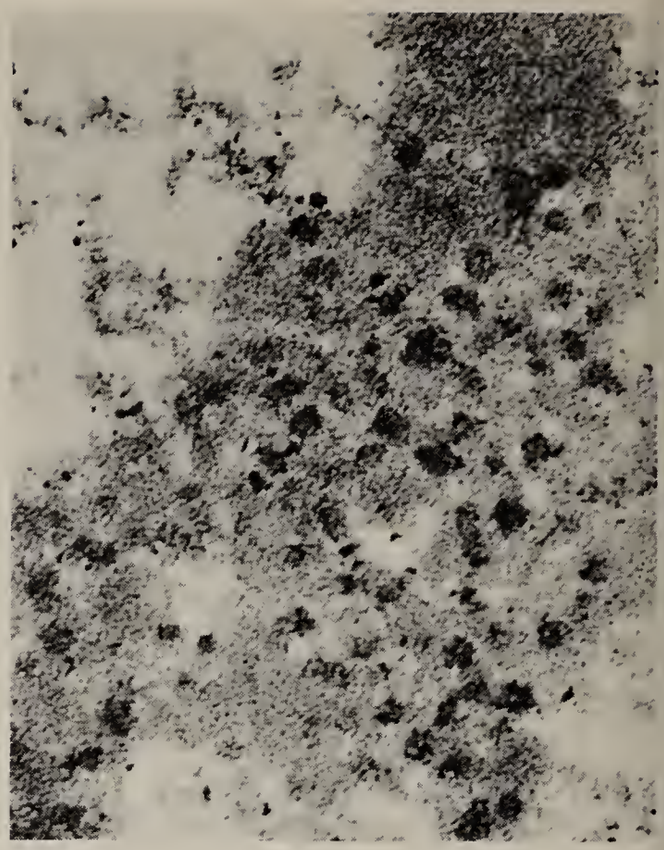


Fig. 12—Large nuclear granules in hepatocyte of chimpanzee with hepatitis A (X 100,000).

REFERENCES

1. **Smetana HF:** The histologic diagnosis of viral hepatitis by needle biopsy. *Gastroenterology 26:612, 1954*

2. **Popper H:** Clinical pathologic correlation in viral hepatitis. The effect of virus on the liver. *Amer J Path 81:609, 1975*

3. **Peters RL:** Viral hepatitis: a pathologic spectrum. *Amer J Med Sc 270:17, 1975*

4. **Klion FM, Schaffner F:** The ultrastructure of acidophilic "Councilman-like" bodies in the liver. *Amer J Path 48:755, 1966*

5. **Schaffner F:** The structural basis of altered hepatic function in viral hepatitis. *Amer J Med 48:658, 1970*

6. **Bianchi L, De Groote J, Desmet VJ, Gedigk P, Korb G, Popper H, Poulsen H, Scheuer PJ, Schmid M, Thaler H, Wepler W:** Morphological criteria in viral hepatitis. *Lancet 1:333, 1971*

7. **Boyer JL, Klatskin G:** Pattern of necrosis in acute viral hepatitis: Prognostic value of bridging (subacute hepatic necrosis). *N Engl J Med 283:1063, 1970*

8. **Baggenstoss AH, Soloway RD, Summerskill WHJ, Elverback LR, Schoenfield LJ:** Chronic active liver disease: The range of histologic lesions, their response to treatment, and evaluation. *Hum Pathol 3:183, 1972*

9. **Popper H, Schaffner F:** The vocabulary of chronic hepatitis. *N Engl J Med 284:1154, 1971*

10. **Popper H, Schaffner F:** Chronic hepatitis: Taxonomic, etiologic and therapeutic problems. Edited by H. Popper and F. Schaffner, *Progress in Liver Diseases, Vol 5.* New York, Grune and Stratton, 1976, pp. 531-558

11. **De Grotte J, Desmet VJ, Gedigk P, Korb G, Popper H, Poulsen H, Scheuer PJ, Schmid J, Thaler H, Uehlinger E, Wepler W:** A classification of chronic hepatitis. *Lancet 2:625, 1968*

12. **Scheuer PJ:** *Liver Biopsy Today.* Edited by F. Schaffner, S. Sherlock, and C. M. Leevy. New York, Intercontinental Medical Book Corporation, 1974, pp. 105-116

13. **Edmonson HA, Peters RL, Reynolds TB, Kuzma OT:** Sclerosing hyalin necrosis of the liver. A recognizable clinical syndrome. *Ann Intern Med 59:646, 1963*

14. **Perez V, Schaffner F, Popper H:** Hepatic drug reactions. Edited by H. Popper and F. Schaffner, *Progress in Liver Disease, vol. 4.* New York, Grune and Stratton, 1972, pp. 597-625

15. **KLION FM, Schaffner F, Popper H:** Hepatitis after exposure to halothane. *Ann Intern Med 71:467, 1969*

16. **Hadziyannis S, Gerber MA, Vissoulis C, Popper H:** Cytoplasmic hepatitis B antigen in "ground-glass" hepatocytes of carriers. *Arch Pathol 96:327, 1973*

17. **Gerber MA, Hadziyannis S, Vissoulis C, Schaffner F, Paronetto F, Popper H:** Electron microscopy and immunoelectron microscopy of cytoplasmic hepatitis B antigen in hepatocytes. *Amer J Path 75:489, 1974*

18. **Huang S-N, Groth V, Beaudoin JG, Dauphinee WD, Gruttmann RD, Morehouse DD, Aronoff A, Grault H:** A study of the relationship of virus-like particles and Australia antigen in liver. *Hum Pathol 5:209, 1974*

19. **Nowoslawski, A Krawczynski K, Nazarewicz T, Slusarczyk J:** Immunopathological aspects of hepatitis type B. *Amer J Med Sc 270:227, 1975*

20. **Shikata T, Uzawa T, Yoshiwara N, Akatruska T, Yamazaki S:** Staining methods of Australia antigen in paraffin sections: Detection of cytoplasmic inclusion bodies. *Jap J Exper Med 44:25, 1974*

21. **Deodhar KP, Tapp E, Scheuer PJ:** Orcein staining of hepatitis B antigen in paraffin sections of liver biopsies. *J Clin Pathol 28:66, 1975*

22. **Huang, S-N:** Immunohistochemical demonstration of hepatitis B core and surface antigens in paraffin. *Lab Invest 33:88, 1975*

23. **Popper H, Schaffner F:** Pathophysiology of cholestasis. *Hum Pathol 1:1, 1970*

24. **Huang S-N:** Hepatitis-associated antigen hepatitis: An electron microscopic study of virus-like particles in liver cells. *Amer J Path 64:483, 1971*

25. **Gudat F, Bianchi L, Sonnabend W, Thiel G, Aenishaenslin W, Stadler GA:** Pattern of core and surface expression in liver tissue reflects state of specific immune response in hepatitis B. *Lab Invest 32:1, 1975*

26. **Edgington TS, Chisari FV:** Immunological aspects of hepatitis B virus infection. *Amer J Med Sc 270:213, 1975*

27. **Barker LP, Maynard JE, Purcell RH, Hoofnagle JH, Berquist KR, London WT:** Viral hepatitis, type B, in experimental animals. *Amer J Med Sc 270:189, 1975*

28. **Provost PJ, Wolanski BS, Miller WJ, Ittensohn DL, McAleer WJ, Hilleman MR:** Biophysical and biochemical properties of CR 326 human hepatitis A virus. *Amer J Med Sc 270:87, 1975*

29. **Deinhardt F, Peterson D, Cross G, Wolfe L, Holmes AW:** Hepatitis in marmosets. *Amer J Med Sc 270:73, 1975*

30. **Schaffner F, Dienstag J, Purcell R, Popper H:** Ultrastructural studies of marmoset livers after infection with human hepatitis viruses A and B. *Unpublished data*

FULMINANT HEPATITIS AND DEVELOPMENT OF ARTIFICIAL LIVER SUPPORT

Roger Williams

During the past ten years we have treated over 200 cases of fulminant hepatic failure in the Liver Unit at King's College Hospital. This figure includes only the most severe cases who develop signs of grade 3 or 4 encephalopathy with stupor or coma (Fig. 1). The most frequent cause was type A infectious hepatitis, but this was a presumed diagnosis only, for, until very recently, as you well know, there have been no specific tests for this variety. Indeed, I have often wondered how this type of hepatitis, which is usually such a benign illness, has taken such a completely different course in these rare cases. Perhaps they represent another completely different virus type.

You may be surprised at the large number of cases which are due to paracetamol (acetaminophen) overdose; in Great Britain would-be suicides seem to have a predilection for this drug. Our anesthetic colleagues are becoming less stubborn, and, hopefully, cases of halothane-induced hepatic necrosis, which have followed multiple exposures in over two-thirds of our cases, will become rarer.

CAUSES OF FULMINANT HEPATIC FAILURE WITH GRADE IV ENCEPHALOPATHY
LIVER UNIT, KING'S COLLEGE HOSPITAL 1966 - MARCH 1976

ACUTE VIRAL HEPATITIS		102
	A 52	
	B 32	
	Unclassified 18	
PARACETAMOL OVERDOSE		80
HALOTHANE ASSOCIATED		25
OTHER DRUG TOXICITY		12
AMANITA PHALLOIDES		2
FATTY LIVER OF PREGNANCY		3
LEPTOSPIROSIS		1
		225

Fig. 1—Causes of fulminant hepatic failure as seen over a 10-year period. In the type B hepatitis cases, HBsAg was detected by immunodiffusion or radioimmunoassay. Sera were not tested in the unclassified cases.

From King's College Hospital and Medical School, Denmark Hill, London, S.E. 5

Roger Williams, M.D., F.R.C.P., Director, Liver Unit and Consultant Physician, King's College Hospital and Medical School.

Whatever the cause, according to worldwide figures, fulminant hepatic failure carries a high mortality of 80 to 90 percent. But recovery, when it occurs, is complete, and in our experience, and in that of the Los Angeles group,[1] cirrhosis does not develop. This is another reason for attempting to treat the condition by every possible means.

This brings me to the use of temporary liver support which can provide time for such recovery to take place. My initial view, and one to which I still adhere despite all the tribulations that have ensued, is that only a completely artificial system of liver support — not one based on perfusion of animals or cadaver human liver — can provide the reproducible and repeatable support that these patients need.

On 23 September 1973 — just over two years ago—we first connected a patient to an artificial liver support system. He was a man of 26 years, in deep coma from fulminant hepatic failure. For four hours that day, and for similar periods on two subsequent occasions, his blood was perfused at a rate of 300ml a minute through a column containing activated charcoal. Following the third perfusion his conscious level lightened and, as you can see from Fig. 2, he subsequently made a complete recovery. The pretty girl with him was in the Unit at the same time, was also recovering from liver failure, and whether it was the common interest or not, they appeared together at the follow-up clinic married, and subsequently had a very fine addition to the family.

The use of charcoal hemoperfusion in this patient had, of course, been preceded by a long series of experimental studies, One to which I would briefly draw your attention was a controlled trial in dogs with liver failure, in which the charcoal-perfused group showed a statistically significant prolongation of survival.[2]

Charcoal was used in the hemoperfusion system because it is a very effective adsorbent for a wide range of water soluble substances, including molecules in the middle molecular weight range (300 to 2,000). Early attempts to use charcoal hemoperfusion were hampered by severe loss of platelets and by the liberation of small particles of charcoal into the circulation. To improve biocompatibility, Smith and Nephew Research Ltd, with whom we have worked closely during the past few years, coated the charcoal with an acrylic hydrogel, the same material as is used for soft contact lenses. The exact thickness of the applied coating is a compromise between biocompatibility and the reduction in adsorptive capacity caused by the coating.[3]

It was the recovery of consciousness from deep coma that was the most striking feature in our initial series of 31 patients treated by charcoal hemoper-

Fig. 2—Clinical photograph of the first patient treated by charcoal hemoperfusion photographed at a later date with his wife and baby, by which time liver function and histology had retumed completely to normal.

fusion.[4] This leads me to a consideration of the biochemical abnormalities underlying hepatic encephalopathy. These are exceedingly complex, with many interactions. Zieve and colleagues,[5] for instance, have shown that the coma-producing potential of toxic substances, such as ammonia, fatty acids and mercaptans, may be multiplied severalfold when they are present together. With respect to the possible correction of such abnormalities by charcoal hemoperfusion, there is evidence from various experimental studies that mercaptans and ammonia may be adsorbed. In addition, the brain may be damaged secondarily by anoxia, so common in hepatic failure, or by hypoglycemia. Progression of these various lesions leads eventually to brain death with the development of cerebral edema in 36 percent of the cases in our series.

Currently there is also a great deal of interest in the relationship between changes in the plasma amino acid pattern, which is known to be grossly deranged in hepatic failure, and the synthesis of false neurotransmitters in the brain. These various precursor amino acids are very efficiently removed by charcoal hemoperfusion Serial measurements in a 32-year-old woman with Amanita phalloides poisoning, who was perfused on four successive days before recovering consciousness, are shown in Fig 3. On each occasion the plasma levels of phenylalanine and tyrosine fell, as did methionine, another amino acid which is increased in the blood of these patients and which may be important in the pathogenesis of the encephalopathy.

The importance of cerebral edema in the progressive cerebral deterioration of some of these patients cannot be disputed. Fortunately we have now an experimental model in which to study the underlying mechanism and the effectiveness of possible treatment measures, such as dexamethasone. In the pig with liver failure induced by surgical devascularisation, we have recently shown that there is no sudden change in intracranial pressure, but a steady and progressive rise (Fig 4). With the rise in intracranial pressure the electroencephalograph showed progressive deterioration, with the tracing becoming flat just before death occurred. Although the development of cerebral edema in man is related, at least in part, to the severity of liver failure, we also have seen a number of patients die from this complication at a time when their liver function was improving.[6]

This aspect is illustrated further in Fig 5 which shows estimates by a morphometric technique, of the percentage of surviving, or remaining, liver tissue in patients dying of fulminant hepatic failure.[6] The hepatocyte volume fraction was lowest in those patients who did not have cerebral edema, hemorrhage, or infection, and in whom liver failure appeared to be the sole cause of death. But in about two-thirds of those who died of cerebral edema or one of the other complications, the hepatocyte volume fraction was higher and within the range where recovery would be anticipated if these complications had not occurred or had been more effectively treated.

An undue susceptibility to bacterial infection characterizes the patient with fulminant hepatic failure, and some of our hemoperfused patients have died tragically of pulmonary infections and septicemias at a time when their liver function was recovering. This brings me to another series of studies,[7] in which we have determined the activity of the hexose monophosphate shunt pathway in leucocytes as an index of bactericidal capability. $^{14}CO_2$ production by the white cells is measured in the presence of normal serum and killed Candida albicans. The mean value obtained for the patient's leucocytes was identical to that of controls, so there is no intrinsic abnormality in the polymorphs. However, when in the assay normal serum was replaced by fulminant hepatic failure serum, there

was a marked drop in activity (Fig 6). Characterization of this inhibitory substance in the serum is currently under way but one other interesting finding which I must mention is that pre-incubation of fulminant hepatic failure serum with the same coated charcoal as that used in our perfusion system will increase leucocyte metabolic activity to normal.

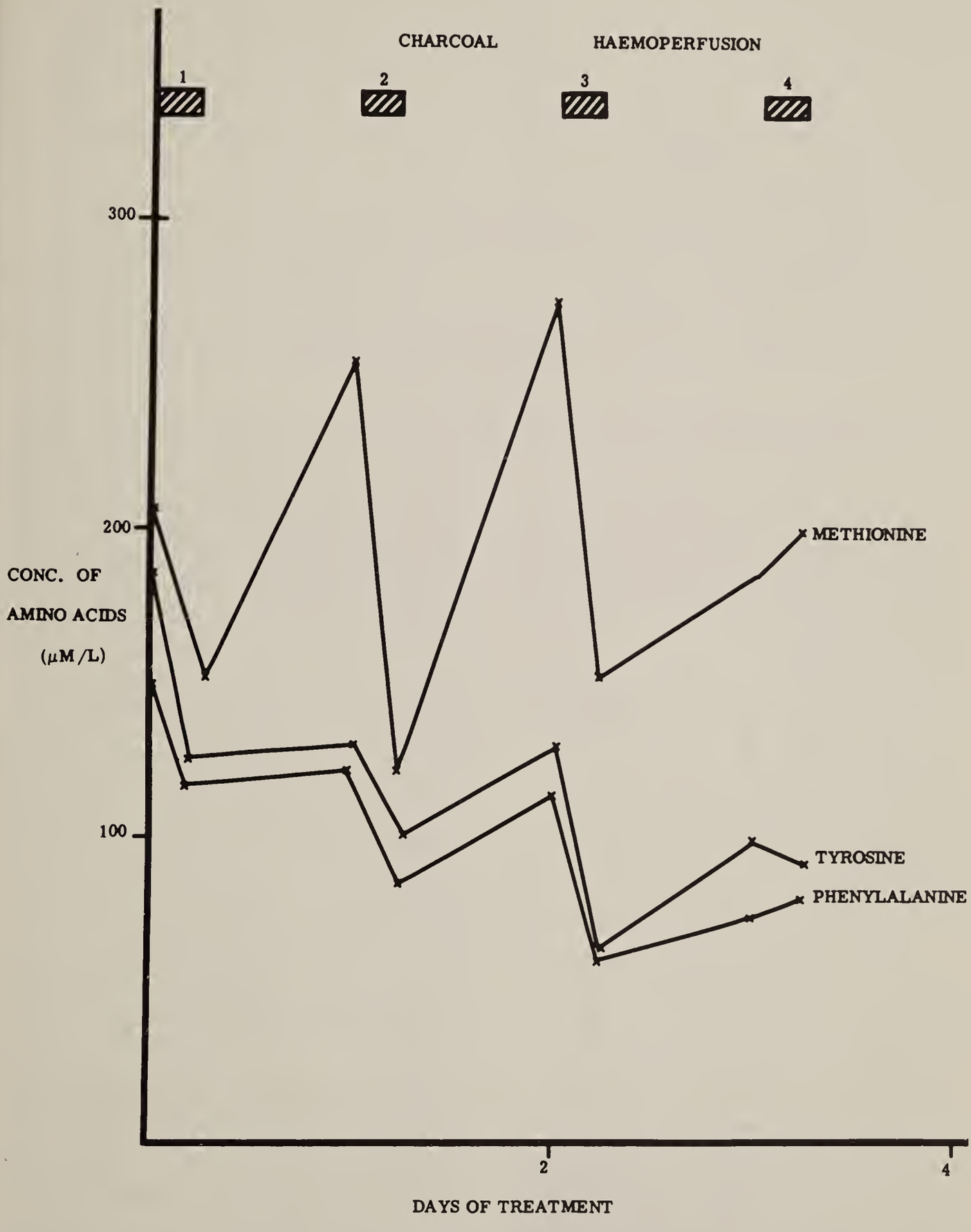


Fig.3—The fall in plasma levels of three amino acids during four periods of hemoperfusion in a patient with hepatic necrosis from Amanita phalloides poisoning (from Gazzard et al., 1974b[4])

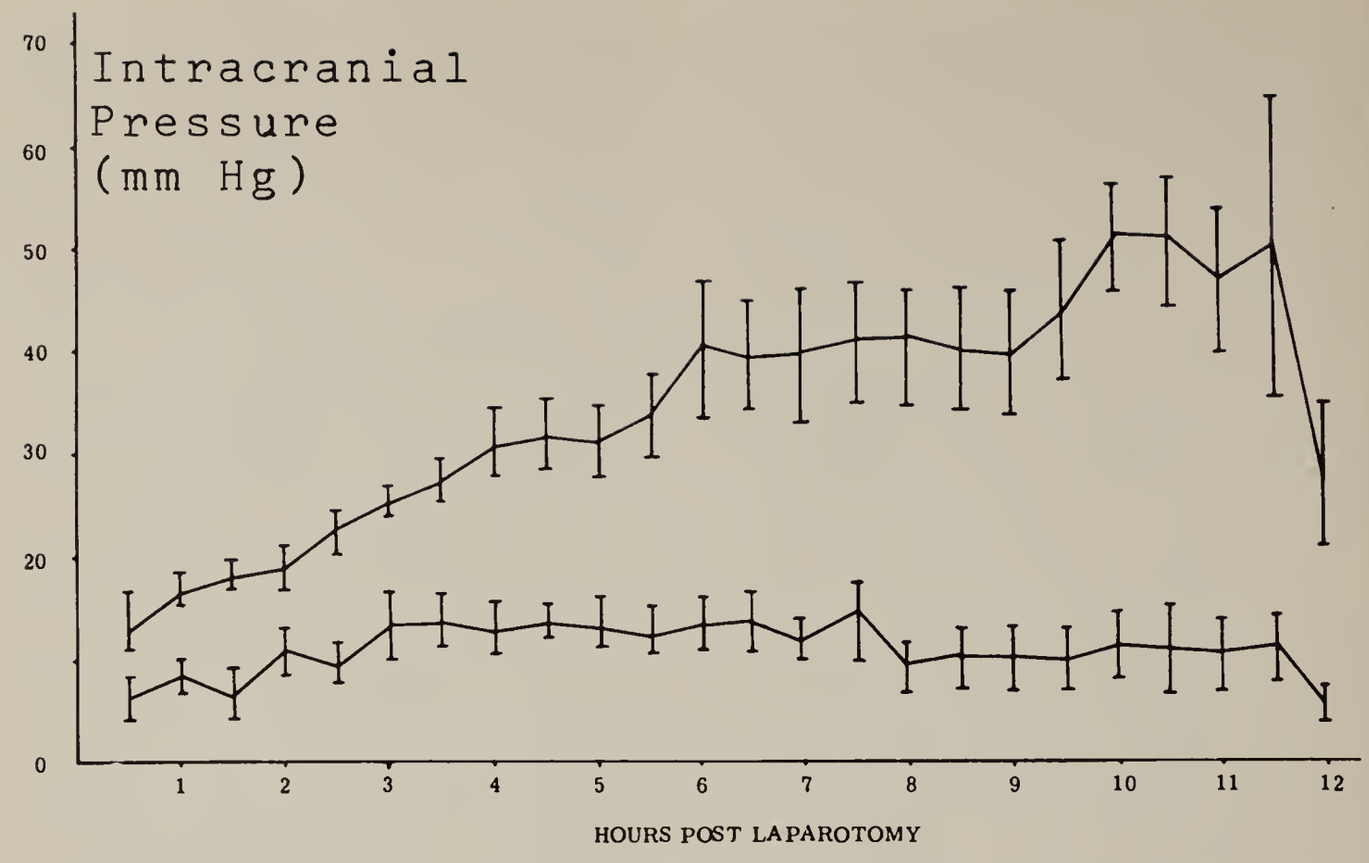


Fig. 4—Intracranial pressure measurements in test and sham operated pigs. Mean values ± SEM are shown (from Hanid et al., 1976).[18]

Whilst on the subject of inhibitory factors, I will touch briefly on another study in which plasma from patients with fulminant hepatic failure has been shown to be cytotoxic to isolated hepatocytes in a tissue culture system.[8] This cytotoxic factor differs from the leucocyte inhibitor in certain respects,

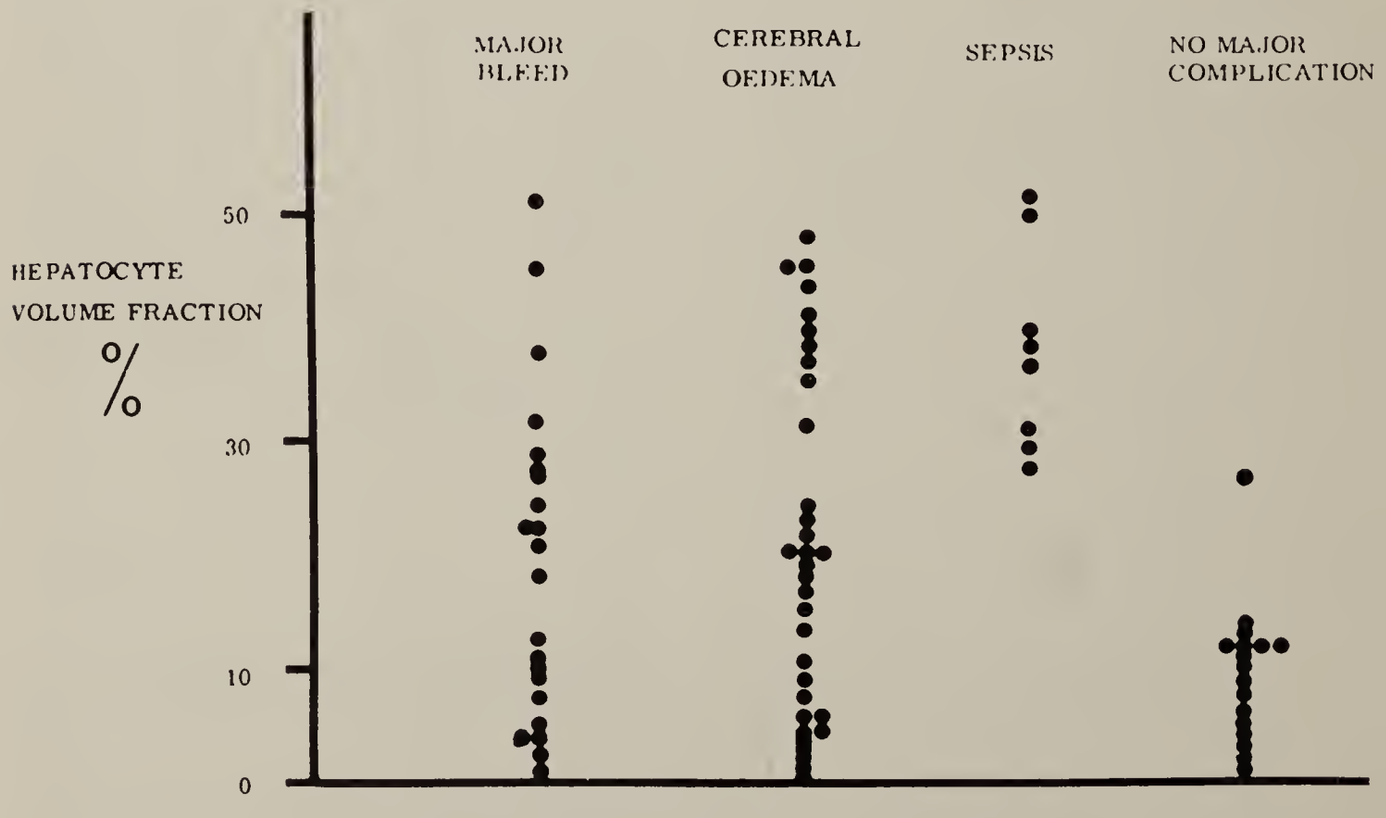


Fig. 5—The hepatocyte volume fraction (HVF) for patients dying from hemorrhage, cerebral edema and infection compared with those in whom hepatocellular failure was the only cause of death. (from Gazzard et al., 1975).[6]

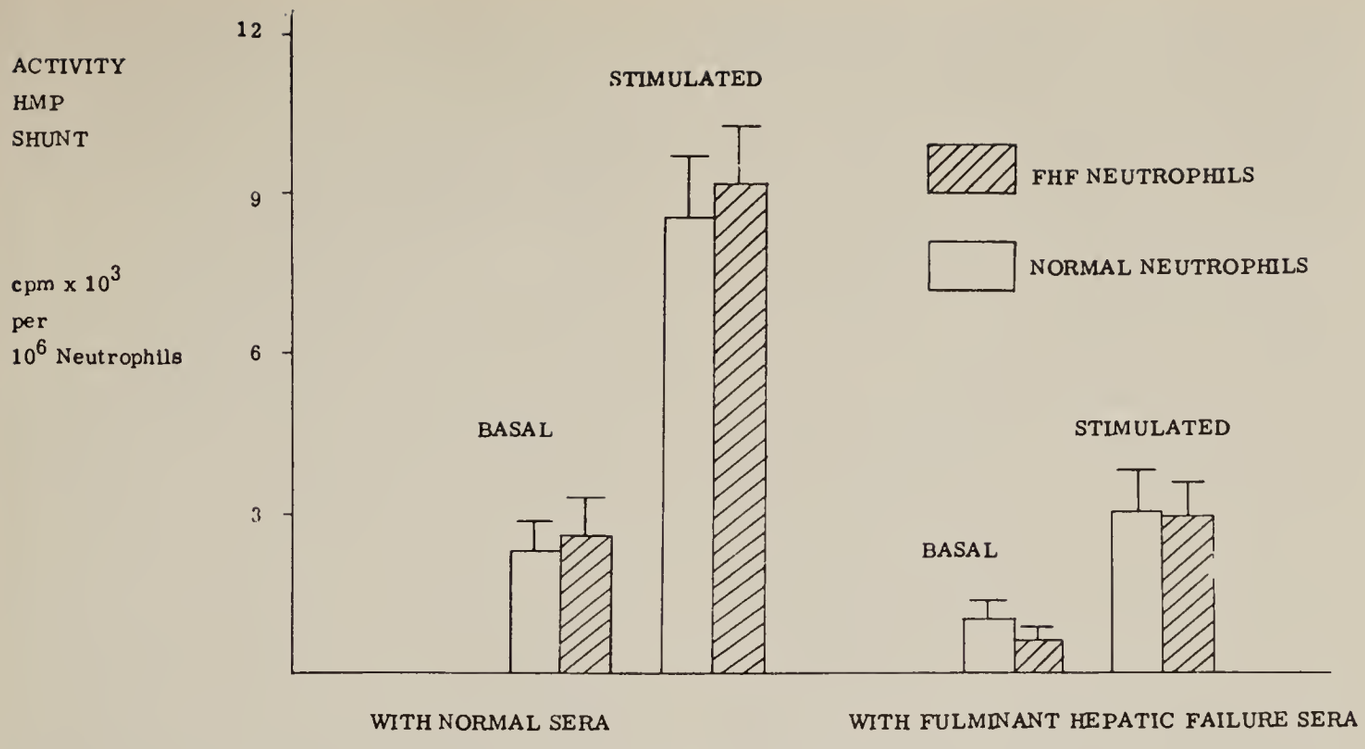


Fig. 6—HMP shunt activity of normal neutrophils and FHF patient neutrophils in the presence of normal serum and FHF serum (mean ± 1 SEM) (from Bailey et al, 1976).[7]

but resembles it in that it is also reduced by charcoal hemoperfusion (Fig 7). The presence of such a factor in the serum may explain why regeneration often appears to be delayed in these patients, and is clearly a most important area for further research. Some degree of hypotension not due to blood loss or other known factors is common in hepatic failure, and it is often assumed that the heart is failing at this stage. However, Trewby has very recently been measuring left ventricular stroke work in relation to levels of pulmonary artery wedge pressure, before or after the infusion of plasma. The line joining these two points represents part of the Starling ventricular function curve. In each of six patients, all of whom were hypotensive at the time of study, the slope was steep, indicating good ventricular function.

Clinically, the hypotension is usually associated with warm peripheries and the immediate cause is probably an opening up of arteriovenous shunts in the periphery and in other organs. For instance, we have shown in recent studies that similar shunts may be present in the lungs.[9] Furthermore, there is

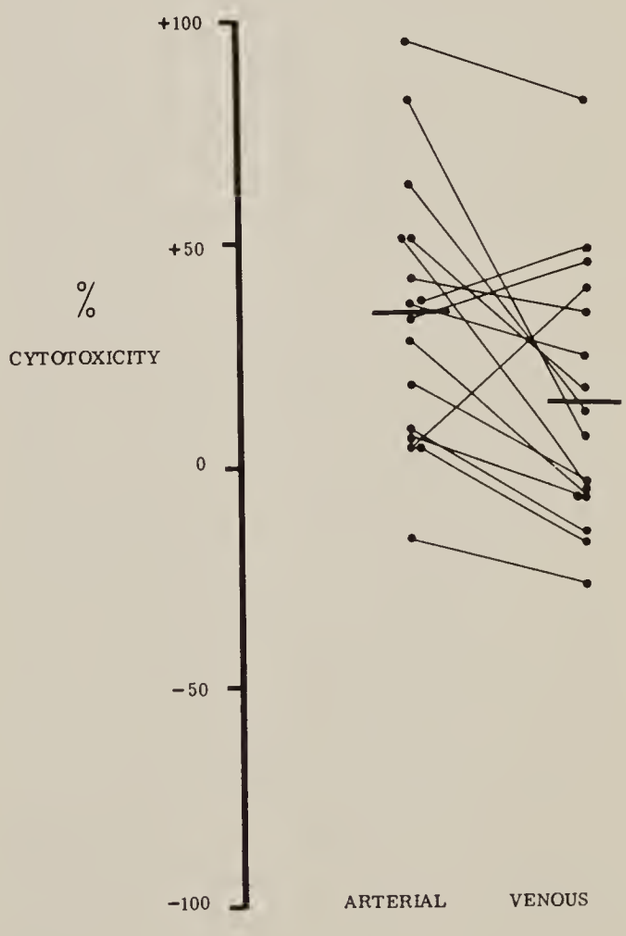


Fig. 7—Percent cytotoxicity of plasma from the inflow (arterial) and outflow (venous) lines of the charcoal column during the first hemoperfusion in 14 patients with fulminant hepatic failure. Results are also shown for samples taken during the second perfusion in two of these patients. The horizontal line indicates mean value in each group.

an excellent correlation between the magnitude of the pulmonary shunt and those of the periphery as shown by arteriovenous oxygen content difference: the smaller the difference the larger the shunt. Pulmonary shunts of up to 20 to 40 percent can be found without any abnormality in the chest radiograph, and sequential studies have shown that if the patient's condition improves they can disappear quite rapidly. Such intrapulmonary shunts are more than sufficient to account for the hypoxia found in these patients and which can have such an important secondary effect on organ function.

One possible explanation for the development of these shunts is endotoxemia. Experimentally in the rat injections of endotoxin are followed by opening of the arteriovenous shunts, with the development of a typical hyperdynamic circulation.[10] We first reported in 1974[11] the occurrence of endotoxemia in fulminant hepatic failure, showing how it was related to the glomerular filtration rate, probably as a result of its effects on the renal circulation. We also found a correlation with intravascular coagulation. Another known effect of endotoxin is damage of vessel walls, with activation of the extrinsic coagulation pathway. The cause of endotoxemia in hepatic failure is an impaired clearance by the hepatic Kupffer cells of the endotoxins normally absorbed from the intestine. Unfortunately, endotoxin levels in the blood do not appear to be reduced by hemoperfusion, although there is one *in vitro* study showing adsorption of endotoxins by charcoal.[12]

Another part of the clinical syndrome of fulminant hepatic failure relates to the hemorrhagic diathesis. In addition to the reduction in platelet count, there is an impaired synthesis of coagulation factors by the liver, and the plasma concentration of these factors is reduced further by intravascular coagulation. But the most important factor determining bleeding is almost certainly the development of localized mucosal erosions in the esophagus and stomach. The erosive esophagitis may be related to reflux of acid gastric contents. Continuous recording of esophageal pH shows episodes of reflux, as evidenced by fall in pH, particularly during turning and physiotherapy. Since gastric acid is at least one factor in the development of gastric and duodenal lesions, we decided recently to use the new H_2-receptor antagonists.[13] So far, cimetidine has been given to four patients bleeding profusely from erosions. Within six hours of starting medication , bleeding stopped and, although it recurred in one patient after withdrawal of the drug, the bleeding again ceased following further treatment. Perhaps more importantly we have also shown that prophylactic use of H_2-receptor antagonists appears to have an important place in the prevention of bleeding in these patients. So far, in a controlled trial (Fig 8), ten of the 18 patients in the control group have had profuse bleeding from either gastric or esophageal erosions. In contrast, bleeding did not occur in any of the 14 who received metiamide or cimetidine sufficient to maintain an intragastric pH greater than 5, a highly significant difference ($p=0.001$).

But I must now return to charcoal hemoperfusion and the overall result. At the time of our first publication in the *Lancet* in June 1974,[4] 31 patients with fulminant hepatic failure had been treated, the survival rate of 39 percent comparing most favourably with our previous figure of 13 percent in 93 patients with grade 4 coma managed by standard supportive measures.[4] But from then on, the results got steadily worse, with no survivals in the last 18 patients perfused, and with a much greater frequency of severe hypotensive reactions (50 percent of cases), the cause of which I shall return to shortly. At that stage, perfusions were stopped, and since then, the biocompatibility of the charcoal preparation has been actively reconsidered.

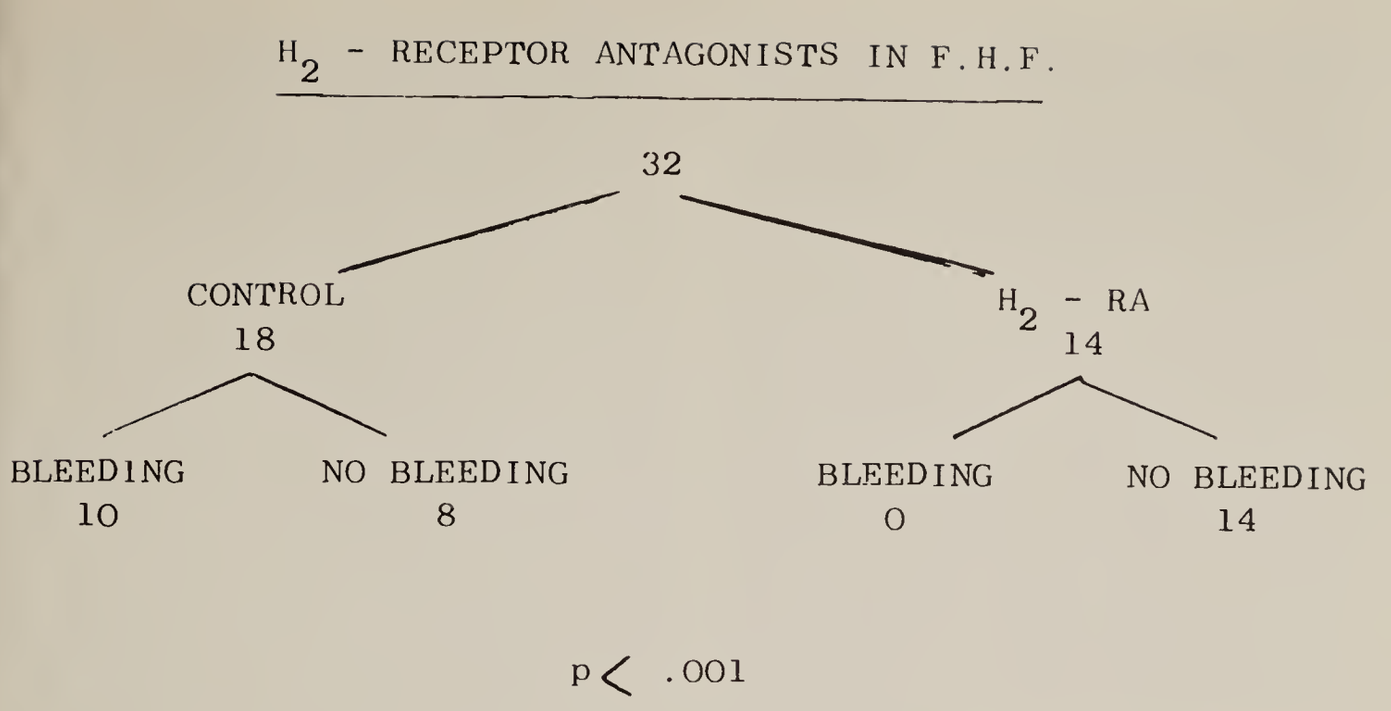


Fig. 8—Results of a controlled trial of cimetidine in prophylaxis of bleeding in patients with fulminant hepatic failure (from Bailey et al, 1976).[13]

Although the hydrogel coating of the charcoal reduces platelet loss, percentage reductions in platelet count over the four-hour period of perfusion were greater than 30 percent of the preinfusion level in over a quarter of the perfusions. Another effect of charcoal hemoperfusion is shown in Fig 9. Platelet sizes in the peripheral blood vary, as can be seen in the distribution curve obtained with blood from a normal subject. In patients with liver failure we found that the curve shifted slightly to the left, even before perfusion, indicating a greater percentage of small platelets in the total count.[14] Following perfusion, the curve shifted even further to the left, presumably because of selective adherence of the remaining larger and stickier platelets to the charcoal. The loss of these larger platelets, which are known to be hemostatically more active than the smaller ones, accounts for another observation, namely, a disproportionate prolongation of bleeding time in relation to the measured reduction of platelet count.

During hemoperfusion we have shown also that platelet aggregates may form within the column and are then released into the circulation.[14] To detect these we have used the Swank screen filtration pressure technique, in which an aliquot of the outflow blood is pumped through a metal screen containing small square pores, and the pressure generated is recorded. When aggregates are present in the blood, there is a sharp rise in filtration pressure. Those perfusions in which aggregates have been detected were associated with a greater-than-average reduction in platelet count but, more importantly, with a profound hypotensive reaction. These were presumably due to liberation of vasoactive amines from the platelets as a result of aggregate formation. The formation of these aggregates may be related to an increase in sheer stress building up at the inlet manifold of the column, but it is not yet clear why this occurred only in the later series of perfusions. During the period of these investigations, the preparation and coating of the charcoal has been under critical evaluation, and we are just about to start perfusions again in man.

We also have been evaluating the completely different type of column manufactured by Becton-Dickinson, in which the carbon particles are immobilized on a support polyester film. This is then wound into a spiral coil and

packed into the polycarbonate housing. Blood flows between the coils of the membrane plates. The advantage of this immobilized charcoal is that there is no coating of the charcoal, and therefore none of the constraints on its adsorptive capacity. It pre-supposes, however, that damage to the platelets during hemoperfusion occurs as a result of agitation with loose particles,

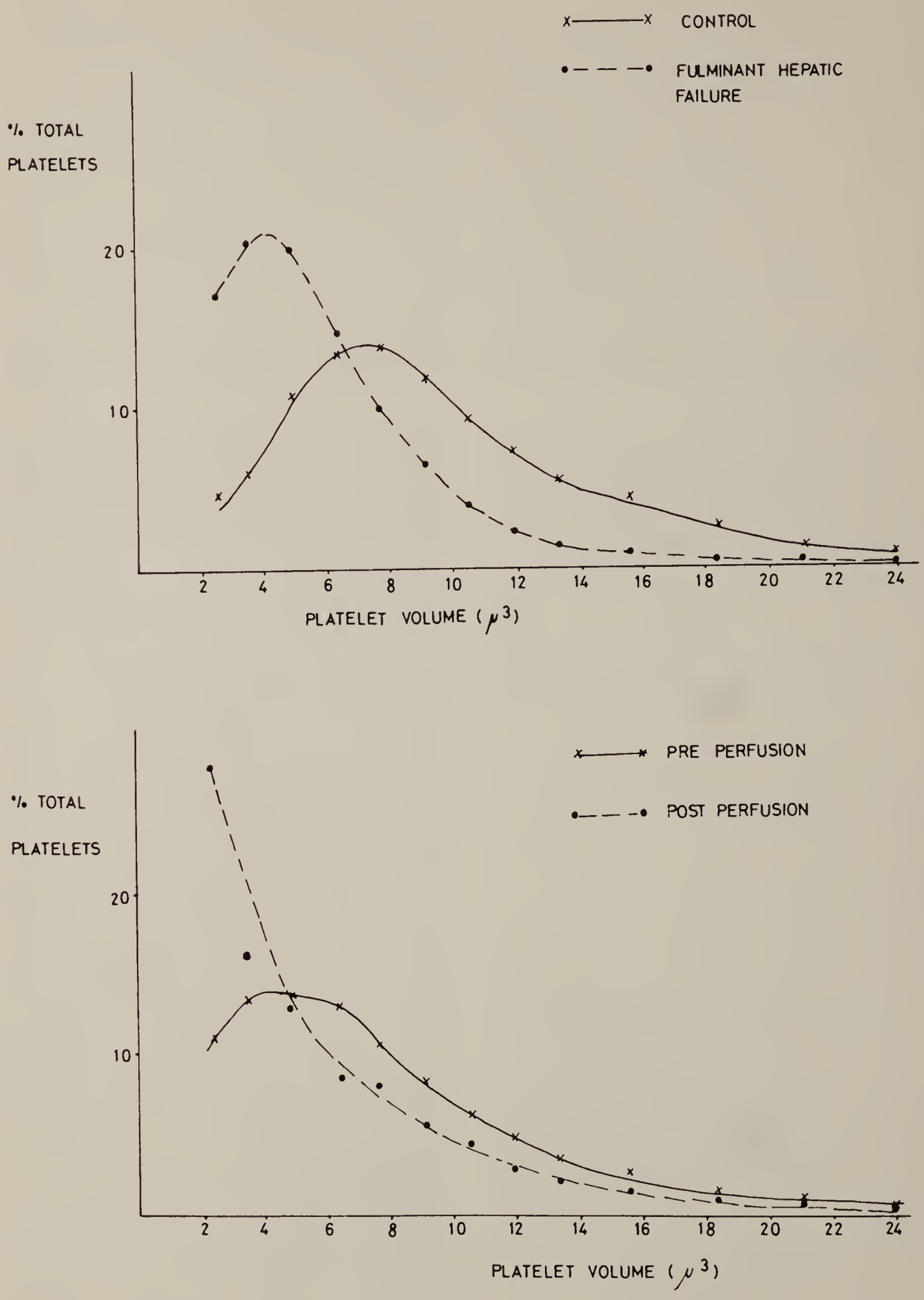


Fig. 9—Platelet population curves from a normal control and patient with fulminant hepatic failure (top) and showing effects of charcoal hemoperfusion (bottom) (from Weston et al, 1976).[14]

which may not necessarily be correct.

Another recent and very exciting development, on which I would like to show you some preliminary data, is the Rhone-Poulenc dialysis machine. This has a novel membrane made of polyacrylonitrile which, unlike the ordinary cuporphane dialysis membrane, allows passage of middle molecular weight substances. It is anticipated that its effect in fulminant hepatic failure would be similar to that of charcoal hemoperfusion. Opolon and colleagues[15] in reporting their experience with this technique describe consistent improvements of conscious levels, although, rather surprisingly, none of the patients treated recovered to leave hospital. Our own experience with it has shown that the removal of amino acids is extremely fast and complete (Fig 10), and two of the patients treated have made a complete recovery. Biocompatibility studies so far would suggest that it is well tolerated.

So far we have been considering only the toxicity of water-soluble substances which can be adsorbed to charcoal and we have not touched on another group of potentially toxic substances, namely bilirubin and bile acids which are bound to the plasma proteins. Several of our patients, one of whom is illustrated in Fig. 11, after recovering consciousness with charcoal hemoperfusion, have passed through a stage of deep cholestasis lasting for several weeks. This particular patient had a halothane-induced hepatic necrosis and was perfused on four occasions. The serum bilirubin subsequently rose to a maximum of 60 mg/100 ml.

Although such protein-bound substances can be efficiently adsorbed *in vitro* to certain resins of the Amberlite series, their biocompatibility is poor, and it is not possible to use them for hemoperfusion. We therefore decided to try a different approach in which the

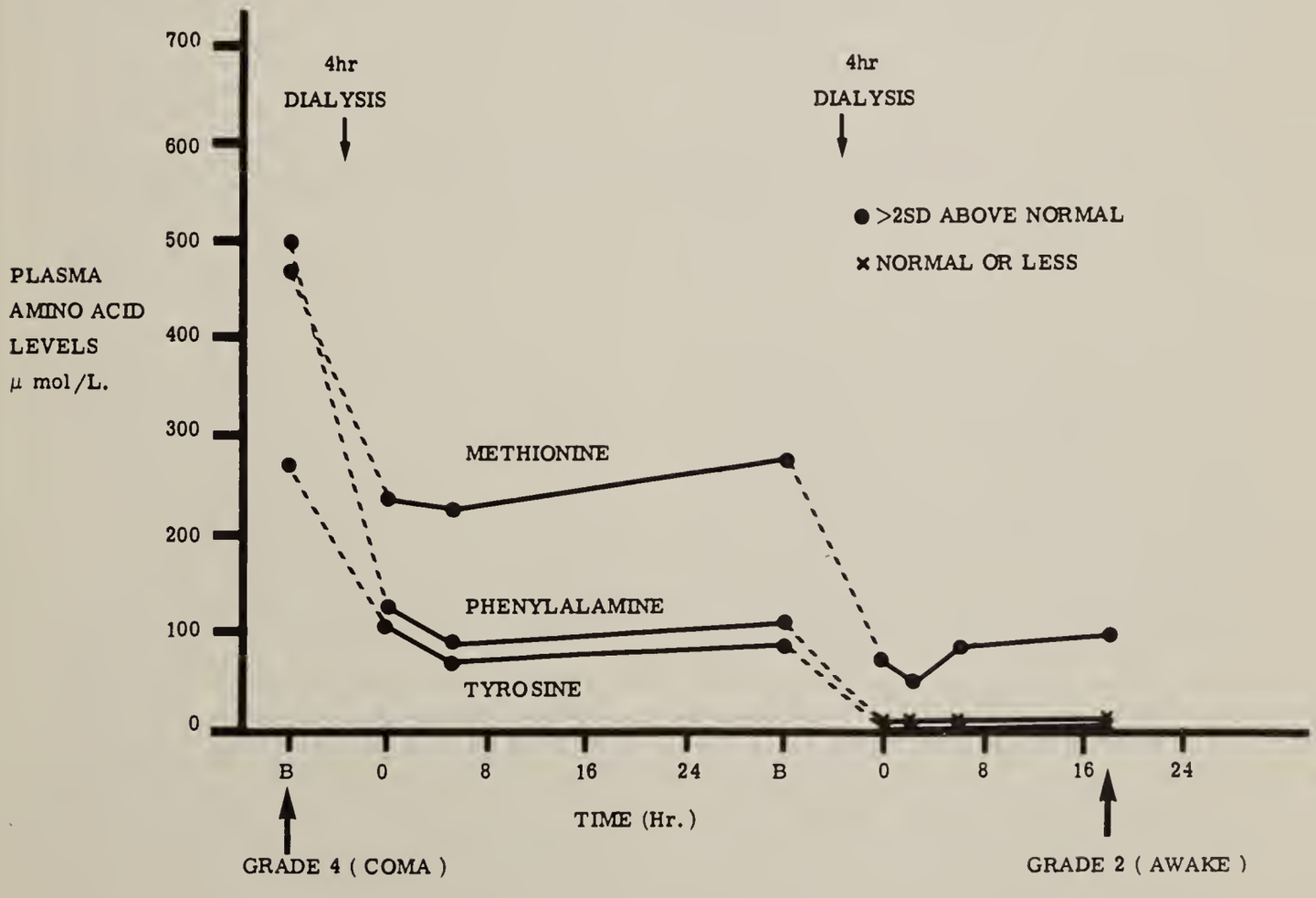


Fig. 10—Serial measurements of plasma amino acid levels during dialysis with the Rhone-Poulenc machine on two occasions in a patient with fulminant hepatic failure (from Silk et al., unpublished data).

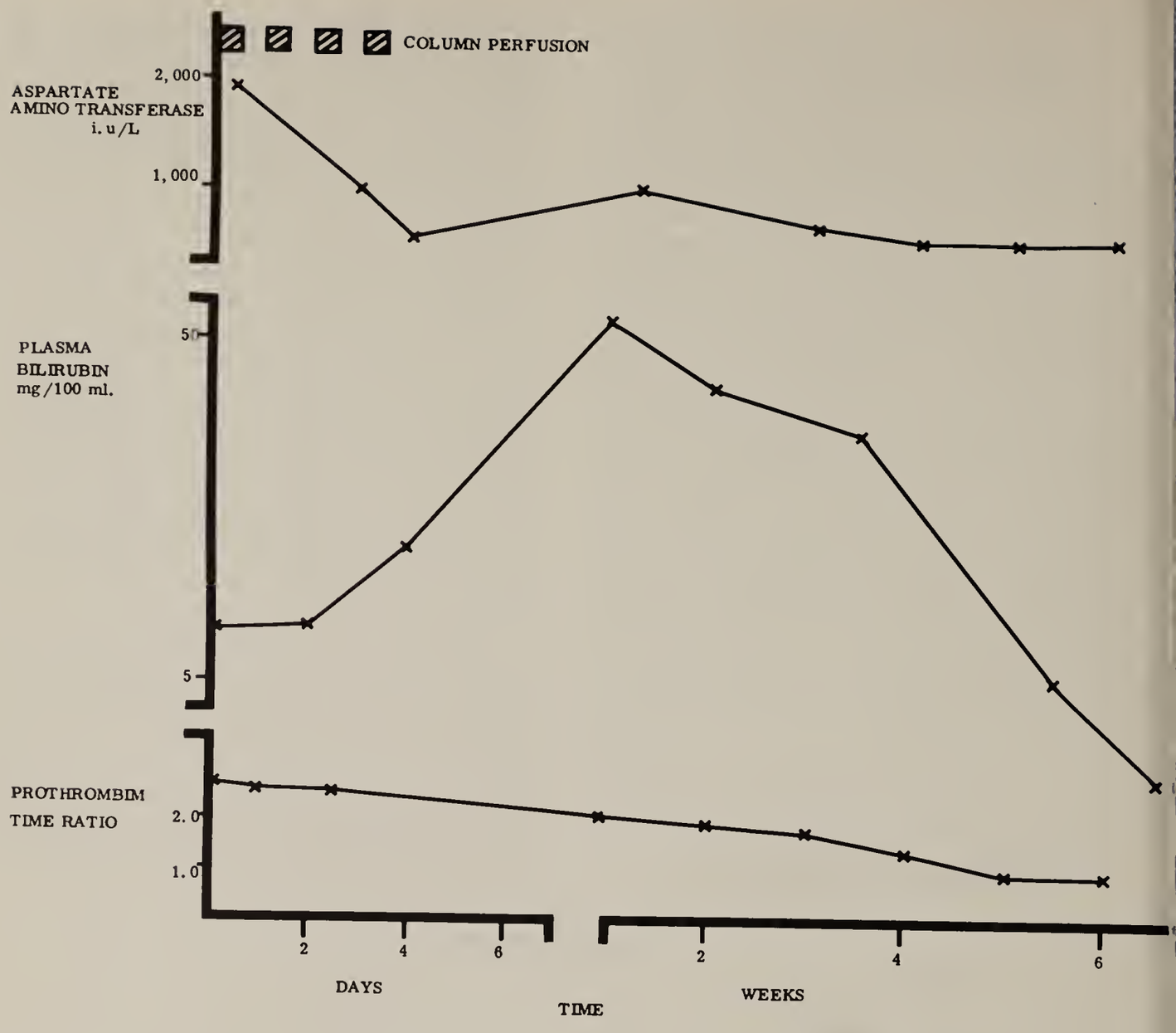


Fig. 11—Serial liver function tests in patient with halothane-associated fulminant hepatic failure who regained consciousness after four hemoperfusions, showing subsequent prolonged cholestatic phase (from Gazzard et al., 1974b).[4]

celltrifuge was used to continuously separate plasma from the formed elements of the blood, the plasma being passed through the columns of XAD-4. The components of the blood are then reconstituted before return to the patient. Unfortunately plasma becomes completely free of platelets only when a very high centrifugal force is used. Even with the lower centrifugal forces that we used and which only partially separated platelets from the plasma, some damage to platelets occurs.[16]

The development of biocompatible and efficient adsorbent/perfusion systems for protein-bound substances is also being pursued by a group at the National Institutes of Health who have developed a very interesting technique in which human serum albumin is bound to agarose beads.[17] The theory behind this is that toxic substances in plasma will dissociate from their binding sites there, and bind, instead, to the albumin covering inert agarose. Our own results with this preparation were rather disappointing, but similar techniques of affinity chromatography can also be applied to other support materials. Currently we are looking at the use of albumin-coated XAD-7. This latter resin, unlike Sepharose, has a high affinity for protein-bound metabolites, so that both supporting

material and coating should contribute to the binding capacity.

How can I summarize all this? The pathophysiology of fulminant hepatic failure is slowly being unravelled. Undoubtedly we learnt much from our first attempts at artificial liver support, and I believe there are going to be many developments here in the not too distant future. Other areas on which I have touched briefly also will need much further exploration, which reminds me of a story attributed to Lord Birkett of a young lady complaining about the Commandments—"they never tell you what to do: they only put ideas into your head." That Richard B. Capps succeeded in doing this over his many years of dedicated service to hepatology there can be no doubt.

ACKNOWLEDGEMENTS

I have drawn heavily on current work of Drs. Bailey, Hanid, Mackenzie, Silk, Trewby, Weston, and Woolf. These studies form part of a long-term program of research into liver failure, supported by the Medical Research Council, the Wates Foundation and Smith's Charity. The National Research Development Corporation and Smith & Nephew Ltd. are also involved in the development of a liver support system, and to all these I am most grateful.

REFERENCES

1. **Karvountzis GG, Redeker AG, Peters RL;** Long term follow-up studies of patients surviving fulminant hepatic failure. *Gastroenterology, 67:870, 1974*

2. **Weston MJ, Gazzard BG, Buxton BH, Winch J, Flax H., Machado A, Williams R:** Effects of haemoperfusion through charcoal or XAD-2 resin on an animal model of fulminant hepatic failure. *GUT 15:482, 1974*

3. **Gazzard BG, Langley PG, Weston MJ, Dunlop EH, Williams R:** Polymer coating of activated charcoal and its effects on biocompatibility and paracetamol binding. *Clin Sci Molec Med 47:97, 1974a*

4. **Gazzard BG, Weston MJ, Murray-Lyon IM, Flax H, Record CO, Portmann B, Langley PG, Dunlop EH, Mellon PJ, Ward MB, Williams R:** Charcoal haemoperfusion in the treatment of fulminant hepatic failure. *Lancet i 1301, 1974b*

5. **Zieve L., Doizaki WM, Zieve FJ:** Synergism between mercaptans and ammonia or fatty acids in the production of coma: a possible role for mercaptans in the pathogenesis of hepatic coma. *J Lab Clin Med 83:16, 1974*

6. **Gazzard BG, Portmann B, Murray-Lyon IM, Williams R:** Causes of death in fulminant hepatic failure and relationship to quantitative histological assessment of parenchymal damage. *QJ Med 176: 615, 1975*

7. **Bailey RJ, Woolf IL, Cullens H, Williams R:** Metabolic inhibition of polymorphonuclear leucocytes in fulminant hepatic failure. *Lancet, 1976, in press*

8. **Hughes R, Cochrane AMG, Thomson, AD Murray-Lyon IM, Williams R:** Plasma inhibitory factors. *Artificial Liver Support,* edited by Roger Williams and I.M. Murray-Lyon, London, Pitman Medical, 1975

9. **Trewby PN, Warren R, Mackenzie R, Crosbie WA, Laws J, Williams R:** "Shock lung syndrome" in fulminant hepatic failure. Proceedings of the British Society of Gastroenterology, *GUT, May 1976*

10. **Liehr H, Grün M, Thiel H, Brunswig D:** Rasenack U: Endotoxin-induced liver necrosis and intravascular coagulation in rats enhanced by portacaval collateral circulation. *GUT, 16:429, 1975*

11. **Wilkinson SP, Arroyo V, Gazzard BG, Moodie, H, Williams R:** Relation of renal impairment and haemorrhagic diathesis to endotoxaemia in fulminant hepatic failure. *Lancet i:521, 1974*

12. **Nolan JP, McDevitt JJ, Goldmann GS.** Endotoxin binding by charged and uncharged resins. *Proc. Soc Exp Biol Med 149:766, 1975*

13. **Bailey RJ, Macdougall BRD, Williams R:** A controlled trial of H_2-receptor antagonists in prophylaxis of bleeding from gastrointestinal erosions in fulminant hepatic failure. Proceedings of the British Society of Gastroenterology, *GUT, May 1976*

14. **Weston MJ, Langley PG, Rubin MR, Hanid A, Mellon P, Williams R:** Platelet function in fulminant hepatic failure and effect of charcoal hemoperfusion. Unpublished data.

15. **Opolon P, Caroli J:** *Etudes des facteurs du traitement des atrophies hépatiques aigues d'origine virale. Médecine et hygiéne, 32:159, 1974*

16. **Weston MJ, Mellon PJ, Langley PG, Hughes RD, Dunlop EH, Gazzard BG, Williams R:** Resin column perfusion with whole blood or plasma separated by the continuous flow celltrifuge. *Clin Sci Molec Med 48:187, 1975*

17. **Plotz PH, Berk PD, Scharschmidt BF, Gordon JK, Vergalla J:** Removing bilirubin and other albumin-bound substances from plasma and blood with albumin-conjugated agarose beads. *J Clin Invest 53:778, 1974*

18. **Hanid MA, Mackenzie RL, Jenner RE, Mellon PJ, Davis M, Williams R:** Raised intracranial pressure and development of cerebral oedema in an animal model of fulminant hepatic failure. Submitted to *GUT,* 1976

NOTICE TO OUR READERS

With this April 1976 issue of Rush-Presbyterian-St. Luke's Medical Bulletin, the publishers regretfully announce suspension of the magazine in its present form.

Established in June of 1962, the Medical Bulletin is currently in its fourteenth year of publication. The Alumni Association and the Editors wish to thank the nearly 6,000 loyal readers whom the Bulletin has served through the years, as well as the editorial staff—editors and assistant editors—who graciously contributed their time to make the Bulletin a periodical of high journalistic and scientific standards. Particularly, we should like to express our appreciation to Dr. Steven G. Economou, who served as the first editor from 1962 through 1968 and to Florence Goodman, Executive Editor since 1968. There has been no flagging of interest in the publication, but funds for continued support of such a private institutional journal are no longer readily available.

Beginning in January, 1977, a semi-annual catalogue of abstracts previously published in national journals, outlining the research efforts of the entire scientific staff of Rush-Presbyterian-St. Luke's Medical Center, will replace the Medical Bulletin as the professional publication of the institution. Until otherwise notified, the mailing list will be maintained intact.

Evan M. Barton, M.D.
Editor